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CONCENTRATION OF MATTER AND ACTION OF ENZYMES IN COACERVATES

by T. N. Yevreinova

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CONCENTRATION OF MATTER AND ACTION OF ENZYMES IN COACERVATES

T. N. Yevreinova

ABSTRACT: The book brings together published data and the author's own experimental findings on coacervate systems consisting of compounds formed biogenically: proteins, nucleic acids, enzymes, carbohydrates, and other biopolymers and low molecular compounds included in the composition of living organisms. Most attention is given to the main property of coacervation, i.e., the concentration of compounds in individua coacervate drops both during their formation and during their absorption of substances, including enzymes, from the surrounding solution. It is shown that coacervate drops constitute very suitable models which can be used to elucidate and reproduce many of the phenomena characteristic of protoplasm, and to approach the solution of the most important problem in biology, the artificial synthesis of living matter.

FOREWORD

The present monograph is a collection of literature data and experimental studies carried out by the author on coacervates obtained from proteins, nucleic acids, enzymes, carbohydrates and other biopolymers and low molecular compounds entering into the composition of organisms and of interest in biology. Coacervates refer to colloidal systems which are best characterized by the concentration of compounds from dilute solutions in the form of liquid coacervate drops of various shapes and structures. /3*

In addition, coacervates have certain properties in common with protoplasm, in which there also takes place an isolation and concentration of matter in coacervate drops.

For this reason, major attention in the monograph has been given to the concentration of compounds in individual coacervate drops both during their

*Numbers in the margin indicate pagination in the foreign text.

formation and during their absorption of substances including enzymes from the surrounding solution. Using coacervates as models, one can gain a deeper understanding of the forms of organization of living matter and its formation from inanimate nature.

In his theory of the origin of life on earth, A. I. Oparin attaches a major importance to the separation of large molecular complexes in the form of coacervate drops from the waters of the primeval ocean. They can thus be regarded as one of the intermediate steps on the path toward life.

The study of this aspect of coacervates is just beginning, and the author will therefore be grateful to the readers for their views and comments.

I take this opportunity to express my sincere appreciation to Academician A. I. Oparin for suggesting the study of coacervates and for the steady interest he has shown during the entire course of the study of this problem; I am also sincerly grateful to Academician T. N. Godney, Academician A. N. Belozerskiy, Professor B. N. Tarusov, Doctor of Biological Sciences S. V. Goryunov, Doctor of Physicomathematical Sciences Ye. M. Brumberg, Professor A. G. Pasynskiy, and senior scientific collaborators I. N. Vlodavets and A. F. Kuznetsova for their helpful comments during the discussion of the manuscript.

It is requested that all the replies and comments be sent to the chair of Plant Biochemistry, Biology and Soil Faculty, Moscow State University im. M. V. Lomonosov. /4

ABBREVIATIONS

TMV - tobacco mosaic virus
RNA - Ribonucleic acid
DNA - deoxyribonucleic acid
NAD - nicotinamide dinucleotide
FAD - flavin adenine dinucleotide
AMP - adenosine monophosphate
ADP - adenosine diphosphate
ATP - adenosine triphosphate
Gum arabic - gum or arabinate
DCPI - dichlorophenolindophenol

Chapter 1

GENERAL CHARACTERISTICS OF COACERVATES

The Concept of Coacervation, and Formation of Coacervates from High Molecular Hydrophilic Compounds

At the end of the 19th and the beginning of the 20th centuries, Kossel, /5
Tiebackx, and Ostwald [748, 845, 931—932] noted that homogeneous, transparent solutions of proteins, carbohydrates and other compounds can separate into two layers, one depleted and one enriched with these compounds. Separation of the matter may occur not only in the form of a layer, but also as liquid "drops", clearly visible under an ordinary microscope (Figs. 1-2) [129]. The terms used for describing this phenomenon were introduced in the 1930's by the well-known Dutch scientist, Bungenberg de Jong, who was the founder of research on "coacervates".

The process of separation into layers was termed coacervation from the Latin word coacervare - to heap up (to cluster). Fig. 3 gives a diagram of this process. The layer rich in molecules of the dissolved substance is referred to as the coacervate layer and the droplets are the coacervate drops; the liquid medium adjoining it is the equilibrium liquid, which always contains less substance than the original solutions.

For instance, if a 0.5% solution of histone is combined with a 0.1% solution of RNA and their total content is taken arbitrarily to be 100%, then of 1 ml of mixture only 0.7% will remain in the equilibrium liquid, while the remaining 99.3% of RNA and histone molecules will concentrate in the drops. The formation of such a system is shown in Fig. 4 [129].

At the present time, two definitions of the "coacervate" concept exist.

According to the first definition, given by Bungenberg de Jong [508 - - 509, 518], "coacervate" designates only the phase enriched with the dissolved substance; however, this phase is also called the coacervate layer or coacervate drops.

According to the second definition, adopted by many researchers [120, 223, /6
254, 255, 784, 785, 835], by "coacervate" is meant the entire system as a whole consisting of two phases, one rich in and one depleted of molecules of the dissolbed substance, i.e., consisting of the coacervate layer plus the equilibrium liquid or the coacervate drops plus the equilibrium liquid [119, 249, 733].

In our view, the second definition is more acceptable, since it reflects the oneness of the entire system. In his works, Bungenberg de Jong also emphasizes that the coacervate, coacervate layer, drops, and the equilibrium liquid constitute an integrated whole, and that a definite equilibrium exists between them

in the distribution of the substances. For this reason, we shall subsequently adhere to the second definition.

Bungenberg de Jong and his coworkers obtained various coacervate systems and studied many of their properties [508, 598, 599, 600-602, 615].

Coacervates are formed from solutions of organic and inorganic compounds, for example from salts of cobalt. sodium silicate and ammonium hydroxide, hexametaphosphate + $CaCl_2$, from polyvinyl derivatives, from solutions of acetylcellulose in benzene [315], chloroform, and other organic solvents, and also from liquids, carbohydrates, proteins, nucleic acids, etc. [191, 290, 293, 422, 482, 651-655, 657-661, 689-690, 762, 764, 788-790, 859].

Hence, "coacervate" is, in a certain sense, a morphological concept [299], 320, 324, 369].

Coacervates may also contain low molecular natural compounds such as amino acids, sugar, etc. Of major importance is the spatial configuration of the molecules, which determines the asymmetry of the protoplasm [9, 96, 175, 189, 401, 424].

In the last ten years, more than 100 different natural compounds have been obtained by an artificial (abiogenic) method from the simple molecules H_2O, CH_4, NH_3 and CO_2 entering into the composition of the primary atmosphere of the earth, under primitive conditions with the influence of radiant energy, heat, and electrical discharges [84, 296, 356, 380, 421, 937, 960]. Of these compounds, the following were isolated and identified: amino acids, carbohydrates, fatty acids, porphyrins and also a series of nonspecific polymers of the type of homopolypeptides (polyproline), homonucleotides (polyadenylic acid), etc. The chemical nature of some of them has not yet been elucidated.

A much more complex method was required for the artificial synthesis of the protein, insulin, carried out in 1964 by Meienhofer et al. This synthesis was made possible by Sanger's identification of the sequence of the amino acids in insulin [323, 410, 411, 419, 802, 958]. Most likely, the appearance of such a specialized hormone protein had already taken place at the level or organisms. /8

The ocean is thought to have been the cradle of life. The waters of the primeval ocean contained the most diverse compounds which could have served as the material for the formation of coacervates prior to the appearance of life on earth.

The conditions of formation and properties of coacervates may change with the chemical nature of the dissolved compounds and solvent from which the coacervate was formed [129].

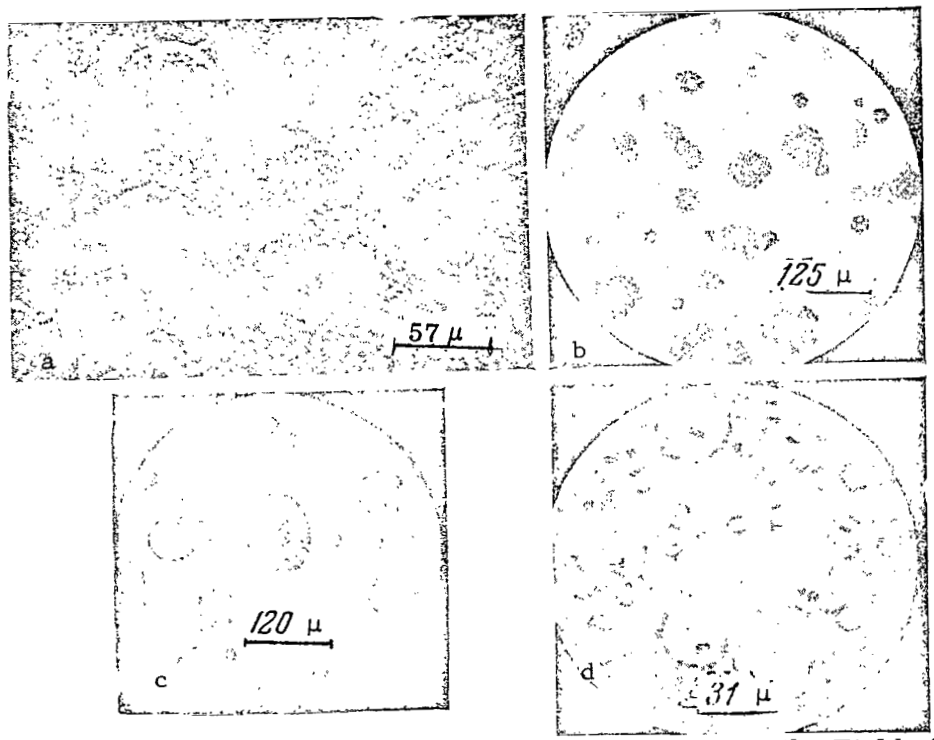


Figure 1. General Appearance of Coacervate Drops in the Field of View of the Microscope (a-d).

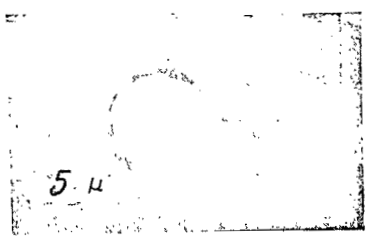


Figure 2. Single Coacervate Drop.

Protoplasm consists mainly of compounds which dissolve well in water. The average composition of protoplasm of various animal and plant cells is as follows: 75-85% H_2O, 10-20% protein (including nucleoproteins), 2-3% lipids, 1% carbohydrates and about 1% salts and other substances [100, 247, 392, 414, 891]. We therefore consider mainly coacervates obtainable from aqueous solutions of natural high molecular compounds: first of proteins and nucleic acids, then carbohydrates and only lipids. The latter differ from the named compounds in many properties. For this reason, lipid coacervates are the subject of a special analysis.

At the time when the theory of "coacervation" was developed by Bungenberg de Jong, aqueous solutions of proteins together with solutions of other compounds, characterized by a high viscosity, a slow diffusion through animal membranes, and many other properties, were classified among colloidal solutions. Thie viewpoint still exists [44, 935]. Kargin et al. [166, 168] who have concerned /9

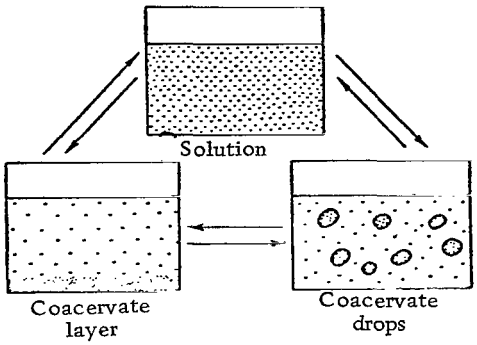


Figure 3. Formation of Coacervates. Point Designate Micells of Colloidal Solutions or Large Molecules of High Molecular Compounds of Proteins, Nucleic Acids, etc.

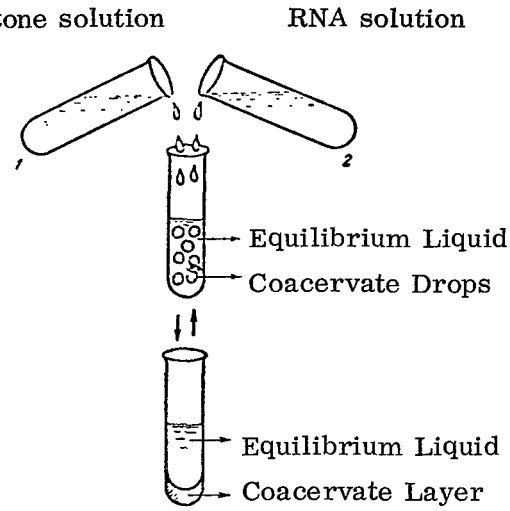


Figure 4. Preparation of Coacervates from Solutions of Histone and RNA at pH 6.5-7.0 and 16-20°

themselves with the study of polymers, have shown that high molecular compounds, and proteins in particular, can give true (mono-molecular) solutions. Because of the large size of the molecules, they retain such properties as viscosity and slow diffusion, which are characteristic of colloidal solutions, but at the same time differ from them in a number of other characteristics.

On the basis of these assumptions, the process of coacervation of high molecular compounds is regarded as the formation of a two-phase system as a result of phase separation. One phase consists of a solution of the high molecular substance in the solvent, and the second, a solution of the solvent in the high molecular compound. The solution richer in the high molecular substance frequently separates in the form of coacervate droplets [92—93]. This phenomenon is associated with a decrease of solubility. The causes of this decrease can vary. The presentation of the principles of colloidal chemistry concerning this subject is beyond the scope of our work and can be found in appropriate textbooks [92-93, 284, 298, 310, 312, 399].

The first theory of formation of coacervates, advanced by Bungenberg de Jong in 1930-1940, was based on the studies of Kruyt and Loeb [191, 192, 206, 628] and derived from the following assumption: 1) water envelopes exist around the micelles of hydrophilic colloidal solutions of proteins; 2) thanks to such a water mantle, the micelles do not stick together. Under the influence of various factors removing the water and thus breaking down the envelopes, the micelles come together, and, depending on the amount of water lost, the gradual transition from a solution to coacervates is observed. The main disadvantage of this theory was to assume the presence of uniform aqueous layers surrounding the protein

molecules. Later, it was hypothesized that only a monolayer of water was present around the protein molecules [311, 315].

It has now been shown that water distributes itself unevenly around a protein. The protein molecule contains certain hydrophilic chemical groups having an affinity for water. Such groups include R-COOH - carboxyl, $R-C\begin{matrix}O\\ \diagdown R\end{matrix}$ - carbonyl, R-OH - hydroxyl, RNH_2 - amine, RNH - imine group, etc. The water molecules in solution may be in the form of dipoles and have a complex structure [467]. The dipoles are attracted by hydrophilic groups. In proteins, the hydrophilic groups are distributed unevenly and differ from one another in their different ability to add water [177, 339, 479].

Some data on the number of water molecules associated with one chemical group are given below. These data were obtained by Pasynskiy, who studied hydrophilic groups by means of ultrasound, and also by other researchers [284, 479]. /10

Hydrophilic group	Number of water molecules per group	Hydrophilic group	Number of water molecules per group
$R-\overset{O}{\overset{\diagup}{C}}-OH$	4	R—OH	3
$R-\overset{O}{\overset{\diagup}{C}}-R$	2	$R-NH_2$	2—3
$R-\overset{O}{\overset{\diagup}{C}}-H$	2	$R-\overset{O}{\overset{\diagup}{C}}-NH$ (in protein)	1
R—NH	2	Glucose residues in starch	3

Thus, the magnitude of hydration depends not only on the chemical nature of the hydrophilic group, but also on its location in the molecule. For example, each carbonyl and imine group is associated with two water molecules, whereas when these two groups form a peptide bond in the protein, they bind only one water molecule.

The protein molecule also contains "dry sites" which are due to the presence of hydrophobic lipophilic groups of the type of hydrocarbon radicals. The latter are characteristic of such amino acids as valine, leucine, isoleucine, etc.

Coacervation is associated with the coming together and concentration of molecules in a smaller volume, by their loss of water, i.e., their hydration and solubility decrease [73, 78, 82, 369, 664].

Upon further dehydration, the coacervates convert into a deposit, and under certain conditions all the stages are reversible [171, 192, 233, 371, 507, 587, 588, 605, 650].

It is commonly assumed that coacervate droplets are formed from solutions. However, the intermediate stages of enlargement of the molecules leading to the formation of droplets have not been established thus far. The microscopic changes involved in this process have not been elucidated either. By studying the formation of various coacervate systems consisting of proteins, carbohydrates, enzymes and nucleic acids, we were able to observe under the microscope a gradual conversion of precipitates into coacervate drops. The process required a definite amount of time and was recorded on color movie film by means of time-lapse microfilming. The filming was carried out by A. M. Kudryavtsev (Laboratory of Special Scientific Mircofilming, Institute of Animal Morphology, USSR Academy of Sciences). The object being photographed was a coacervate of gum gelatin, which was chosen as the best-studied system (coacervates with gelatin have recently been used very widely [627]). 0.67% aqueous solutions of gelatin and gum were combined in the ratio of 5:3 and acidified with 4% acetic acid to pH 3.5-4.0. The acidification caused the separation of a flocculent precipitate which changed into drops on heating to 40°. The formation of droplets from the precipitate required 30-40 min. During a single experiment, 960 frames were taken which clearly showed the continuity of the transformation from shapeless flocs of precipitate to droplets [122-125, 259]. /11

Figure 5 shows some individual film frames. The first frame gives an idea of the appearance of the precipitate, in the second frame the precipitate begins to change, and the third shows future drops. An accidental dirt particle proved to be a good orientating mark. Two droplets shown in the fourth frame formed around it. If the heating of the mixture is continued to 60-70°C, the drops disappear by dissolving, and as the temperature is lowered to 20° the drops change into a precipitate from which they can form again at + 42°. /12

Soudek [358] observed the formation of coacervate systems from precipitates obtained from biological liquids. He dried aloe juice, then dissolved it in water. Upon acidification of the solution, flocs precipitated which produced drops after heating.

Thus, coacervate drops can be formed first from solutions and second from precipitates of organic compounds. Bernal [464-465] showed that the synthesis and concentration of organic compounds in the form of precipitates could take place between layers of aluminum silicate clays found in ocean and sea lagoons. The precipitates then migrated into the lagoons, went into solution and gave coacervates [35].

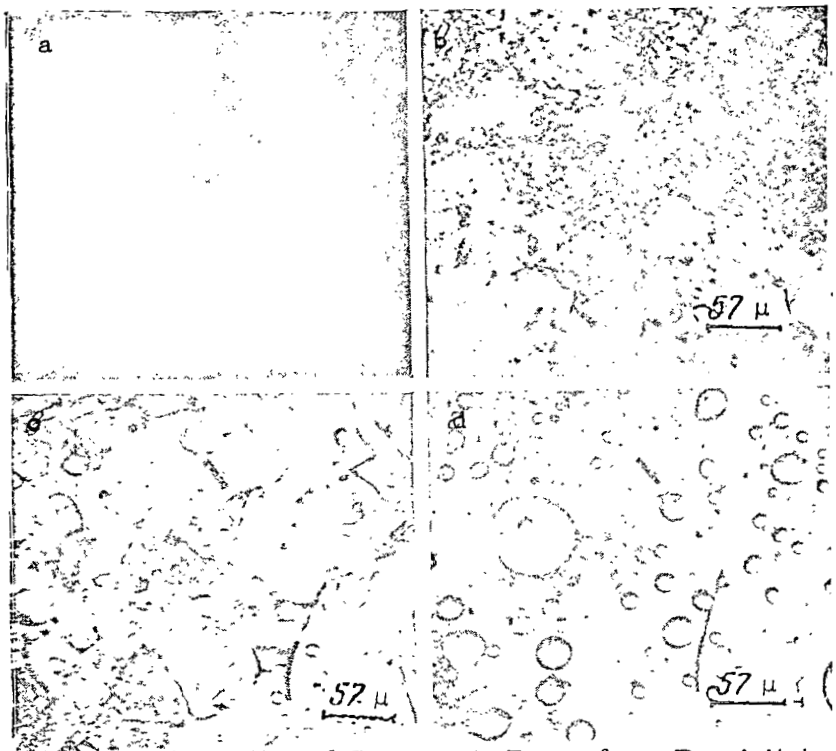


Figure 5. Formation of Coacervate Drops from Precipitates.

a—precipitate, b—start of formation of drops, c—continuation of the process of formation of drops, d—coacervate drops.

Consequently, when coacervates are formed from precipitates, a dilution of the precipitates takes place; the latter swell and produce drops. If the drops are formed from solutions, a concenration of molecules in the drops is observed The process of coacervate formation is represented as follows:

solutions $\rightleftarrows$	microcacervates	$\rightleftarrows$ coacervates $\rightleftarrows$ deposits
10^{-7}—10^{-5}cm high-molecular compounds, colloids	10^{-5}cm	10^{-1}—10^{-2} cm

Upon coalescing, particles of colloidal solutions or molecules of true solutions - proteins, carbohydrates, nucleic acids, lipids - can be converted into very fine droplets tenths of a micron in size, which constitute micro-coacervate systems and practically have not been studied. According to

present day concepts, when polymer solutions are combined, only round drops should be formed at first. It is sometimes possible to see how such droplets fuse together and form larger drops of various shapes. The size of coacervate drops is measured in microns.

On standing, as the temperature changes, for instance on cooling, the drops are converted into layers or into a flocculent precipitate frequently called a flocculate.

Flocculates can be reconverted into drops and solutions [15, 225, 508, 512, 572, 671, 752-755, 947].

Such reversibility is related to the conditions under which the coacervate was formed and its chemical compositon. For instance, if the composition of a coacervate includes serum albumin, the number of conversions is limited. It is usually equal to three or four, and is followed by an irreversible denaturization of the protein. Thus, definite limits of the reversibility exist [593].

Protoplasm, regarded by many authors [44, 285, 508] as a microcoacervate or coacervate, consists of a large assortment of proteins and other compounds having various properties. This is partly why the reversibility of the protoplasm is still more limited than that of coacervates. Protoplasm loses its capacity for reversibility under conditions where many other coacervates retain it. /13

The existence of coecervates also depends on the ratio of the forces of attraction and repulsion operating between the molecules [90, 115, 178, 275, 412, 466, 791]. The energy of these forces and the range of their influence have been analyzed in detail by Bernal and other authors [33, 39, 295, 466] and are given in Table 1.

All molecules are characterized by van der Waals forces of attraction. Their magnitude is inversely proportional to r^6, where r is the distance between the molecules. These forces are of three kinds: 1) those acting between two oriented dipoles; 2) between dipoles arising from a foreign compound - induction forces; 3) between dipoles formed instantaneously as a result of the synchronized rotation of electrons in two molecules. In the last case (3), such forces are called London forces. If there are few or no polar groups in a molecule, but there are hydrocarbon radicals present, the attraction between them is accomplished by London forces [368]. /14

The coacervates are composed of high molecular compounds which readily form hydrogen bonds. Furthermore, they are greatly affected by van der Waals forces, expecially in the case of liquid coacervates, and also polar forces forming a surface charge and then a double layer of ions on molecules and micelles [90, 91, 177, 202, 275, 389].

TABLE 1. Forces of Interaction of Two Particles

Forces	Energy, kcal/mole	Distance between particles, 10^{-3} cm	Example
	Short-range action		
Homeopolar	500	1—2	Atoms having lost an electron (all organic compounds)
Coulomb attraction	20	2—3	Oppositely charged particles (atoms, radicals) halides, R-NH_3^+ and R-COO^-, etc.
Hydrogen bond	15—5	2.4—3.2	OH and NH groups with OH and CO groups, water, acids, sugars, purines, nucleic acids, proteins, etc.
Van der Waals	0.013—9 and more	3—4	All molecules
Cryohydrate (bound by a thin layer of water of oppositely charged ions)	5	3—20	Molecules with a diameter about 10^{-8} cm, protein crystals, bentonites
	Long-range action		
Polar ionization forces of large amphoteric molecules (little-studied)	<1	20—3000	Large-sized ampholytes measuring 100 x 10^{-8} cm and more in water and ionic solutions. High polymer molecules (coacervates, tactoids, gels)

All these forces depend on certain chemical groups and their spatial distribution in the molecule. For example, the hydrogen bond in the carboxyl group of R-COOH, OH----O, has an energy of 8.2 kcal/mole and operates over a distance of 2.7 Å, and in the group R—NH_3NH- - - -N its energy is equal to 1.3 kcal/mole and the distance is 3.38 Å. When the spatial arrangement of the R-NH_2 amine and R-COOH carboxyl groups is close, it is impossible to measure the electrostatic forces, since the hydrogen bonds interfere with the measurement. Only when these groups are distant from each other does the action of electrostatic forces of attraction between amine and carboxyl groups become real. Therefore, it is necessary to consider the configuration of the molecules. Molecules can approach one another, but cannot form even a temporary bond because of the lack of spatial compatibility. All the figures in the table pertain

to interactions between two molecules or radicals without taking external effects into account.

Molecules are usually surrounded by other partners, and thus all the relationships between them and the calculations of the acting forces become very complex [39, 177].

The larger the molecules, the greater the distance at which they can interact. However, the strength of the bond formed is easily impaired.

In addition to forces of attraction, there are also forces of repulsion. These include Coulomb (electrostatic) forces acting between molecules of like charge and also hydration forces. The coacervation is most complete when the forces of repulsion are considerably weakened and the forces of attraction are sufficiently large. A major part in this process is played by hydrophobic radicals of molecules, but the most important role is played by polar groups. Coacervates are /15
formed best by combining solutions of opposite charge [40, 199, 472, 597].

For example, the coacervate of RNA and histone exists at pH 6.5-7.0. In this pH range, the particles of the RNA solution carry a negative charge, and those of histone, a positive charge. The magnitude of the forces of attraction (i.e., charges) between them determines the distribution of the substances (RNA and histone) between the equilibrium liquid and coacervate drops or layer.

Overbeck and Voorn [847, 848, 949—951] have given general forms of mathematical equations which can be used to calculate the approximate distribution of electric charges and substances in the following cases of coacervation:

1. Polyelectrolyte + low molecule ion.

2. Polyelectrolyte + polyelectrolyte, one of which is charged positively and the other negatively; for example, the solutions of $(\text{gelatin})^+$ and $(\text{gum arabic})^-$ at pH 3.3-4.0 and 40°.

3. Two electrolytes with opposite charges + low molecular ions (cations and anions); for example, solutions of $(\text{gelatin})^+$ and $(\text{gum arabic})^-$ and ions K^+ and Cl^- at pH 3.75 and a temperature of 40°.

A more detailed description in the above-mentioned studies is given for coacervation involving two oppositely charged polyelectrolytes. It is shown that in this case the coacervation process is associated with the concentration of charges in the coacervate layer. When the positive and negative charges interact, the free energy of the system decreases. It should be noted that all the conclusions and derivations of the equations are illustrated primarily with relatively simple examples involving the use of gelatin, gum arabic and mineral salts.

It is true that the investigators assumed that the results of the calculations would apply to other systems as well. However, in multicomponent coacervates obtained from diverse chemical substances, the interrelationships between the distribution of charges will obviously be more complex. The use of cataphoresis considerably facilitates the elucidation of the conditions of coacervate formation.

Basic Physicochemical Properties

Cataphoresis. In order to obtain coacervates, one of the essential steps consists in measuring the cataphoresis rate of the solutions at various pH values. The dependence of the cataphoresis rate and pH for 0.67% solutions of gelatin and gum is shown in Table 2 and Fig. 6.

The data of Table 2 show that the higher the absolution product of the cataphoresis rates, the more coacervates are formed, and the optimum pH value necessary for coacervation can be found from the value of the cataphoresis rate [547, 519, 566, 607].

TABLE 2. Rate of Cataphoresis of Solutions of Gelatin and Gum Arbic at Various pH's (in arbitrary units)* /16

pH	Gelatin	Gum Arabic	Absolute product of the ~~rays~~ of cataphoresis of gelatin and gum arabic x 10^{-4}	
4.70	+70	−846	5.9	
4.40	+225	−830	18.7	
4.00	+410	−775	31.8	
3.80	490	−755	37	
3.50	+595	−695	41.4	Coacervates are formed in greatest quantities
3.30	+665	−645	42.9	
3.00	+715	−735	51.5	
2.80	+705	−440	31	
2.50	+665	−280	18.6	
2.20	+618	−170	10.5	

*100 units = 0.24 sec/v per cm at 100-120 V.

The cataphoresis rate changes as a function of the initial concentration of the colloidal solutions from which the coacervate was obtained. When 1% solutions of gum arabic and gelatin are employed, the cataphoresis rate is equal to 473, and in the case of 4% solutions of these substances, to 295 arbitrary units.

Cataphoresis can be observed in protoplasm and coacervates. The rate and direction of the motion of the drops toward the positive or negative pole in cataphoresis may change with the magnitude and sign of the charge on the coacervate. A charge can be changed if one takes different proportions of oppositely charged

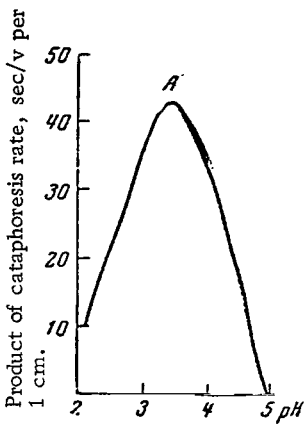


Figure 6. Cataphoresis Rate and pH During Formation of Coacervate from 0.67% Solutions of Gelatin and Sodium Arabinate.

A—Point of Coacervate Formation.

solutions from which the coacervate is to be obtained. For instance, coacervate drops prepared at pH 3.5 and 42° from 0.15% solutions of gum arabic and gelatin containing 83.3% gelatin and 16.7% gum arabic by volume are charged positively. In the presence of 33.3% gelatin and 66.7% gum arabic, the drops will be charged negatively.

The same phenomenon is observed in a coacervate consisting of sodium arabinate and a complex cobalt salt (cobalt hexol nitrate). When the concentration of cobalt hexol nitrate is raised from 11.5 to 12.5 milliequivalents, the charge on the coacervate is reversed, the direction of movement of the drops changes, and the entire coacervate becomes negatively charged and moves toward the anode. The charge reversal of a coacervate is also possible when the pH of the medium changes. The coacervate from gum arabic and gelatin at pH 3.82 is charged negatively, and at pH 3.32, positively [520, 540, 550 577, 583]. /17

In the end, after a long action of direct current, the coacervates break down into the component substances. The coacervate from gum arabic and gelatin separates into gum arabic and gelatin. Gelatin collects at the cathode, and gum arabic at the anode [876,877]. Before the coacervate drops break down a number of morphological changes take place. At the start of the action of the current, the drops flatten and assume the shape of discs.

This flattening has been termed the Buchner effect. It was first observed on coacervates obtained from sols of iron salts and arsenic sulfide [500,508].

If the action of the current was brief, the discs resumed the shape of drops at the end of this action. The degree of deformation of the drop depends on the size of the drop and on the voltage of the direct current. The larger the size of the drop and voltage, the more pronounced is the deformation of the drop. For coacervate drops consisting of gum arabic and gelatin (pH 3—4, 42°, 30 V), the shape of the drops does not change; at 40 V, slight changes are noted, and at 60 V the drop begins to change into a disc [508]. As the current is passed, the disc-like drops also continue to change. The coacervate drops may be charged both positively and negatively. Therefore, the direction of the morphological changes will also be different, depending on the sign of the charge [513, 555, 608]. As indicated in Fig. 7, such a sequence in the change of the shape of the drops can be observed in a coacervate obtained from gum arabic and gelatin. However, /18

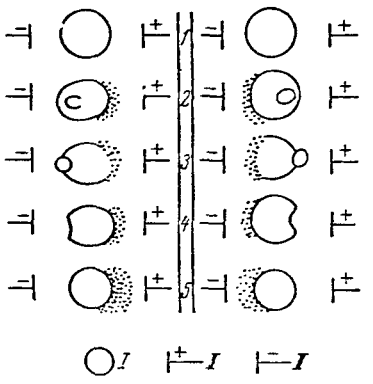


Figure 7. Action of Direct Current on Coacervate Drops. I—Coacervate Drops of Gum Arabic and Gelatin; II—Anode; III—Cathode; 1-Original drop (Start of the Action of Current); 2—Formation of Vacuole Inside Drop, and on the Opposite Side, of a Fringe of Fine Droplets; 3—Displacement of Vacuole to the Periphery of the Drop; 4—Detachment of Vacuole, Enlargement of Droplet Fringe, 5—Drop Becomes Smaller than the Original Drop.

this sequence is not present in all coacervates. In coacervate drops consisting of gelatin and nucleic acid or gelatin and egg albumin, no disclike shapes are formed. There is no vacuole separation in drops formed from solutions of gum arabic and clupein. A halo of fine droplets is lacking in the coacervate from clupein and gelatin. The coacervate from lecithin and Na trioleate in cataphoresis breaks down into fine droplets at first, and then disappears altogether. If KCl or NaCl is added to such a solution which is heated for a long time, the coacervate is reformed [481, 514, 562].

Thus, the chemical nature of the coacervate has a substantial influence on the morphological transformations of the drops when they are acted upon by a direct current. If the coacervate is obtained from hydrophilic compounds and an organic substance such as benzene is added, the benzene penetrates the drops. As the current is passed, the benzene droplet moves to the periphery of the coacervate drop.

A rotary motion is observed inside a charged drop as the current is passing. In a more complex drop having double layer and a vacuole, the direction of the rotary motion remains unchanged.

The morphological changes arising in coacervates under the influence of direct current as well as the appearance of vacuoles, holes around drops, and rotary motion are characteristic of protoplasm as well. Therefore, a certain analogy can be drawn between the behavior of protoplasm and coacervates in an electric field [716].

Viscosity and Surface Tension of Coacervates. One of the characteristic properties of protoplasm and solutions of biopolymers is their high viscosity [95, 363].

Coacervates are heterogeneous liquid systems with an inhomogeneous distribution of substances. Consequently, the viscosity of a coacervate layer or drops will differ markedly from that of an equilibrium liquid.

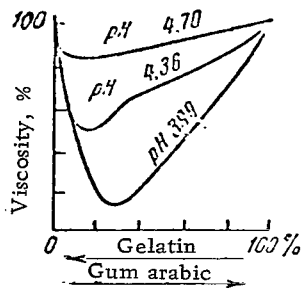


Figure 8. Viscosity of Solutions and Formation of Coacervates at Various pH's (Initial Viscosity and Concentration of Gelatin and Gum Arabic Solutions Taken as 100%).

The viscosity of the equilibrium liquid is low. /19 It is less than the viscosity of the solutions from which the coacervate was obtained. For example, the relative viscosity of a 0.67% solution of gum arabic is 0.344, and that of gelatin (of the same concentration), 0.452 at pH 4.0 and 42°. After the coacervate drops are formed, the relative viscosity of the equilibrium liquid decreases by a factor of five.

The most complete formation of coacervates is observed in the zone of the lowest viscosity of the equilibrium liquid.

Of considerable influence on the viscosity is the pH of the medium and also the concentration and relative amounts of the initial solutions from which the coacervate is formed. The relationship between the factors is shown in Fig. 8 [549, 550, 564, 574].

The formation of precipitates from coacervates is accompanied by an even greater decrease of viscosity. Table 3 shows data on the change in the viscosity of alcohol solutions of zein (a corn protein).

The slight viscosity of the equilibrium liquid, close to that of water, is characteristic of all coacervates consisting of proteins, nucleic acids and other biopolymers.

TABLE 3. Viscosity of Zein Solution as a Function of Alcohol Concentration

Amount of alcohol, %	Relative viscosity	Visible changes in solution
89.7	1.153	Transparent opalescent solutions
80.1	1.153	Same
71.1	1.158	Same
55.1	1.142	Same
40.2	1.085	Coacervate
26.0	1.025	Precipitate

Figure 9 shows the change in the viscosity of solutions of these compounds as as a function of the conditions of formation of coacervates.

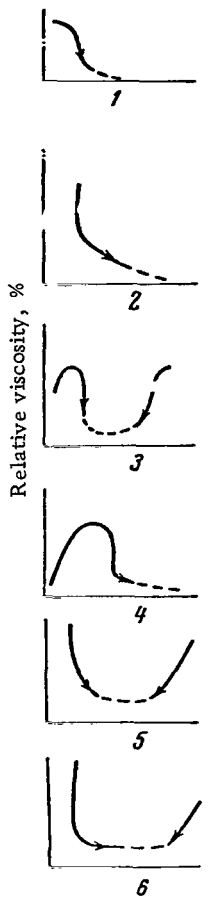


Figure 9. Change in Viscosity of Solutions During Formation of Coacervates under the Influence of Various Substances and pH's; Broken Line - Zone of Coacervate Formation.

1—Alcohol; 2—$MgSO_4$; 3—pH; 4—Picric Acid; 5—Polyolefins; 6—Hexol Nitrate.

The decrease of the viscosity of the equilibrium liquid results from the decrease in the total volume of the particles, since they concentrate into larger coacervate drops.

The viscosity of a coacervate solution calculated from Einstein's formula is valid without additional corrections when the particles have a spherical surface. When the particle change, for example, from spherical to rod-shaped while keeping the same volume, the viscosity increases. In this case, the linear shape of the particle offers more resistance to the current than the spherical shape. At the isoelectric point, when the viscosity is at a minimum, the protein molecules in the solution strive toward a spherical shape owing to the mutual attraction of the molecules due to the equality of the positive and negative charges. As the distance from the isoelectric point increases, the molecules unfold, and the viscosity increases. Staudinger [238-239, 298, 758] has proposed a formula for calculating the viscosity of solutions. However, this formula also requires a number of corrections. /20

The viscosity of a coacervate layer or drops is higher than that of the equilibrium liquid surrounding them and higher than that from which the coacervate was obtained.

The viscosity of coacervate drops or layer depends on the concentration of the solutions used for preparing the coacervate. As the concentration of the initial solutions rises, the viscosity increases. For instance, in the case of 0.01-0.1% solutions, the viscosity of the coacervate layer is 5-15 times that of water. The viscosity of coacervate layers obtained from 2-10% solutions is hundreds of times that of water.

Pchelin and Solomchenko studied the viscosity of a coacervate layer prepared from a 10% solution of gelatin and Na_2SO_4. The gelatin content of the layer was 31.5%.

For comparison, they took a gelatin jelly with the same protein content as in the coacervate layer.

Figure 10 shows that heating causes a decrease of the viscosity of both the coacervate layer and solution. However, the coacervate layer is more stable to the action of temperature. This great thermal stability of the coacervate layer is attributed by the authors to a change in its structure under the influence of Na_2SO_4]307].

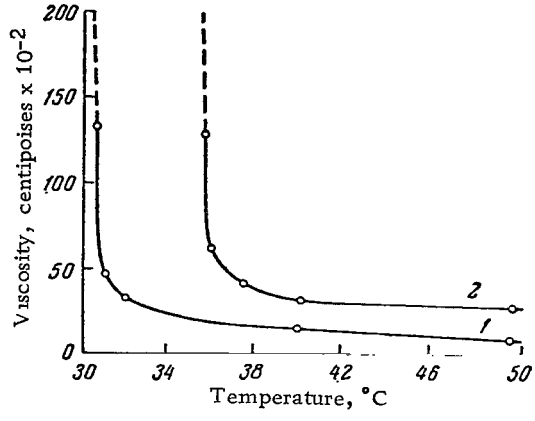


Figure 10. Viscosity (Centipoises) as a Function of Temperature.

1—31.5% Gelatin Solution; 2—Coacervate Layer (gelatin + Na_2SO_4).

An increase in the concentration of the solutions from which the coacervate is formed leads to an increase of not only the viscosity but also the total volume occupied by the coacervate layer. For example, the volume of a coacervate layer obtained from 1% solutions of protein and carbohydrate was 5.31% of the total volume of the solution, and 26.2% when 4% solutions of these substances were employed. The volume of the coacervate layer, particularly if small, can be determined by measuring its refraction [476] and calculated from the formula

$$V_c = 100 \cdot \frac{h_m - h_c}{h_c - h_e},$$

where V_c is the volume of the coacervate layer, h_c is the refraction of this layer, h_e is the refraction of the equilibrium liquid, and h_m is the constant of refraction of the solution containing no coacervate.

By measuring the refraction of the coacervate one can determine whether a dilution or concentration of the coacervate layers takes place under the influence of various chemical and physical factors.

Some comparative data on the viscosity of the protoplasm of cells and other items follow [89, 100, 238, 284, 376].

Water has a viscosity of 0.8937 centipoises; milk, 1.7; human blood plasma, 1; human blood, 3-4; nerve fiber, 5.5; ameba (Ameba dubia), 2-14; ovum (Arbacia punctulata), 7; slimy molds, 9-18; Chara fragalis, 10; paramecium, 50 centipoises.

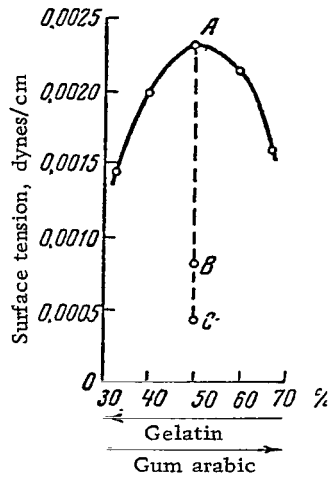


Figure 11. Surface Tension Between Coacervate Layer and Equilibrium Liquid in Protein-Carbohydrate Coacervate. On the Abscissa Axis, the Initial Concentration of Gum Arabic and Gelatin Solution is Taken as 100%. A) Optinum point of coacervate formation; B) 7 meg of KCL added; C) 10.5 meg of KCL added.

The existence of coacervate drops or layer is determined by the surface tension arising at the interface between the drops and the equilibrium liquid. If the surface tension is insufficient, the drops dissolve. The magnitude of the surface tension depends on the proportion of the components from which the coacervate was obtained and also on extraneous compounds added to the system [855]. Figure 11 shows the change in the surface tension for a coacervate consisting of gum arabic and gelatin and added KCl. /22

An increase of the KCl concentration leads to a decrease of the surface tension. This should be kept in mind, since in cells a major part is played by salt, which obviously can also affect the surface tension of individual structures.

In a living organism, the surface tension existing between various liquid forms of the protoplasm and between the protoplasm and the cell fluid is of major importance. Some data [120, 376, 394] on the surface tension of the protoplasm of living organisms in contact with various liquids are given below.

It is known that the surface tension at the water-air interface is equal to 72-73 dynes/cm. For leucocytes (Ringer's solution + Blood serum) it is 2.0, for amoeba (Ringer's solution), 1-3, for myxomycetes of Tphysarium polycephalum (Ringer's solution diluted by a factor of 250), 0.45, for ova of Arbacia punctulata (sea water) 0.2, for ova of Triturus viridescens (pond water + gum arabic), 0.1. Certain coacervate drops or layers (equilibrium liquid) have a surface tension of 0.0025-2.31.

When cells burst, the protoplasm on passing into water assumes the shape of globules resembling coacervates. A certain definite surface tension exists between the globules of protoplasm and water, just as between the coacervate drop and the equilibrium liquid surrounding it. It turns out that the amoeba, leucocytes, and coacervate drops or layers have similar surface tensions relative to the liquid surrounding them. /23

The preceding brief survey of the physical properties of coacervates shows that the properties of coacervates and protoplasm have much in common: behavior in an electric field, presence of vacuoles and process of their formation, generation of rotary motion, viscosity close to that of plasma, surface tension - all of which makes protoplasm similar to coacervates. However, coacervates differ fundamentally from protoplasm not only in the lack of any sign of life but also in the fact that the chemical composition of prepared model coacervate systems is still very simple. In most cases, they consist of a small number of different substances and frequently contain homogeneous, unstructured drops. In some cases, they are true liquids.

For true liquids, the rate of flow is proportional to the force applied, whereas for protoplasm no such relationship exists [74, 318]. When various structural formations arise within a coacervate drop, they lose the properties characteristic of true liquids, even despite the simplicity of the chemical composition.

Depending on the complexity of their chemical composition and hence on their properties, coacervates can be divided into simple ones and complex ones [481].

Chapter 2

TYPES OF COACERVATES

Classification /24

The physicochemical classification of coacervates now in use was given by Booij and Bungenberg de Jong in 1956 [481].

The basis of the classification are the polarity sign and magnitude of electric charges of the molecules.

All the coacervates are divided into the following categories:

I. Simple ones.

II. Complex ones.

1. Single-complex ones.

2. Two-complex ones.

3. Three-complex ones.

Simple coacervates are formed by the dehydration of hydrophilic solutions, which leads to a decrease of their solubility [508].

Thus, simple coacervates cover molecules of the same chemical composition. The formation of complex coacervates is associated with an interaction between the positive and negative charges of the molecules [487].

Depending on the charge which they carry, particles of solutions of high molecular compounds are conventionally called amphoteric ions when the amounts of positive and negative charges are equal, and macroions if they are charged positively (macrocation) or negatively (macroanion). Microions consist of low molecular compounds, chiefly mineral salts.

The interaction of charges in these groups is shown in Fig. 12.

In single-complex coacervates, the positive charges of one amphoteric ion are attracted to the negative charges of another, and vice versa, amphoteric ions being represented by the same chemical compound. A coacervate is formed particularly easily from molecules of proteins of phosphatides at the isolectric point. /25

Two-complex coacervates arise from the interaction of two oppositely charged compounds.

This may include the following variants: macrocation + microanion; macroanion + microcation; macroanion + macrocation.

The latter case is the most common. This is the method used to prepare coacervates from alkaline + acid proteins, phosphatides + proteins, proteins + RNA or DNA, etc. For instance, at pH 6.0, the particles of histone are charged positively, and those of RNA negatively, and on mixing they give a coacervate.

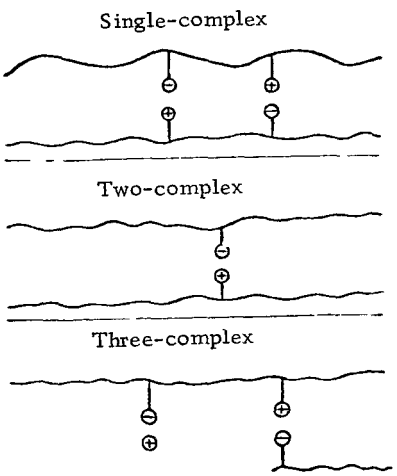
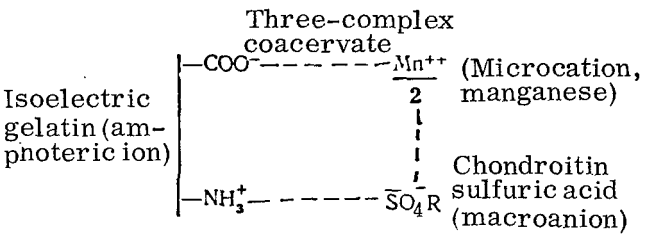


Figure 12. Interaction of Charges in Solutions During Formation of Coacervates (after Booij and Bungenberg de Jong).

I—The same Amphoteric Compound (macroamphoteric ion); II—Two Different Compounds (macroions); III—Two Different Compounds (one macroamphoteric ion, the other a macroion) and a Microion.

Three-complex coacervates are fairly complex systems. They are formed by combining an amphoteric ion + macronion (macrocation or macroanion) + microion (cation or anion). An example of such a type of coacervate is gelatin at the isoelectric point + potassium chondroitinsulfuric acid + $Mn(NO_3)_2$. The interaction of charges in this coacervate can be represented as follows:



Other combinations are also possible in theory. K^+ can replace Mn^{++}, and the radical of chondroitinsulfuric acid can be replaced by NO_3^-. However, no such coacervates were obtained. There are also other discrepancies between the classification and what actually takes place. For instance, there is a certain subgroup of auto-complex coacervates whose formation involves the participation of particles surrounded by a double ionic layer. According to Bungenberg de Jong, this layer is formed by adding salts mainly to hydrophilic solutions of phosphatides. /26

According to present-day concepts, molecules of proteins and phosphatides in hydrophilic solutions are surrounded by a double ionic layer without the necessity of adding salts. The salts already present in the solutions are sufficient for the formation of ionic layers. Hence, the presence of a double ionic layer also takes place in other cases of coacervation, although its size may vary.

Coacervation may be accompanied by the formation of new complex chemical compounds [311, 500, 564, 578, 583—581, 591—592, 614, 616] i.e., nuclear proteins, lipoproteins, and glycoproteins, frequently called biocomplexes [36, 109, 112, 158, 269, 272], etc.

Oparin and Bardinskaya have shown that serum albumin and gum arabic are present in the coacervate as glycoproteins [256].

In the last few years, coacervates have been obtained from various proteins, enzymes, low molecular organic compounds, salts, etc., all these compounds being in the same coacervate. For example, the synthesis of starch was accomplished in a coacervate of the following composition: histone, gum arabic, phosphorylase enzyme, glucose-1-phosphate, NaF, CH_3COONa, starch. Fairly complex interrelationships of charges arise in such a system [129, 208].

All these deviations from the classification should be considered in studying coacervates.

There is not doubt that the physicochemical classification is suitable, particularly for coacervates with a small number of well-studied components such as gelatin, gum arabic, etc. Obviously, in further treatment it will be possible to use the physicochemical classification for more complex coacervates. Thus far, however, it is more convenient for our purposes to consider both simple and complex coacervates depending on the number and chemical nature of the compounds comprising them (components).

Simple coacervates. The properties of simple coacervates were studied most thoroughly in connection with their preparation from aqueous gelatin solutions [457, 564, 656]. When a neutral salt such as sodium chloride or sulfate, which removes water from the gelatin molecules, is added to such solutions, the latter separate into two layers, and a coacervate is formed (on heating to $+50°$). The amount of gelatin in both the coacervate layer and the equilibrium liquid may change and depends on the total gelatin content of the solution from which the coacervate was formed.

For example, if a solution contains 3% gelatin, then in preparing the coacervate 2.02% of gelatin will be found in the coacervate layer and 0.98% in the equilibrium liquid. When the concentration of the gelatin solution is changed to 1%, the coacervate layer will contain 0.93% gelatin and only 0.07% of the latter will be present in the equilibrium liquid. A definite relationship exists between the /27

gelatin content of the coacervate layer and of the equilibrium liquid. The less gelatin there is in the coacervate layer, the less of it is present in the equilibrium liquid. The lower limit at which the formation of a simple coacervate is still possible is a 0.001% concentration of gelatin in the solution. In order to obtain a coacervate from such a dilute solution, it is necessary to add 24.6% sodium sulfate, and the mixture must be heated to +50°. The maximum formation of coacervate takes place when a 7.5% solution of sodium sulfate and gelatin is used. ' If different amounts of sodium sulfate, alcohol or resorcinol are added to the aqueous solutions of gelatin of the same concentration, the coacervate layer will occupy a different volume in each individual case. Interesting properties are displayed by the coacervate obtained from aqueous solutions of gelatin and resorcinol. Gelatin dissolves much better in resorcinol than in water, and it therefore migrates into the layer containing the most resorcinol. Comparison of the properties of simple coacervates from gelatin and the conditions of their preparation are given below.

Composition of Coacervate and Its Properties

Aqueous gelatin solution + alcohol or + sodium sulfate	Aqueous gelatin solution + resorcinol
A large amount is required	A small amount is required
Gels on cooling	Does not gel on cooling
Gelatin insoluble in Na_2SO_4 and in alcohol	Gelatin soluble in resorcinol
The coacervate layer contains less sodium sulfate and alcohol than the equilibrium liquid	The coacervate layer contains more resorcinol than the equilibrium liquid.

Such compounds as resorcinol, phenol, pyrocatechol, hydroquinone, pyrogallol, hydroxyhydroquinone, and phloroglucinol readily form simple coacervates with proteins at 40° [505, 845].

Simple coacervates can be obtained not only from gelatin, but also from other proteins. Coacervates are formed from solutions of amandin (globulin from almond seeds) in the course of dialysis in cold water (the coacervate dissolves on heating) and also from alcohol solutions of prolamines when they are diluted with water; from alkaline solutions of protamines when alcohol is added to them. A /28
solution of clupein sulfate (protamine from herring) on cooling gives an oily coacervate layer which dissolved on heating. This can also be observed in the case of salmine (protamine from salmon) [656, 749, 840].

Some dyes, for example, trypaflavine, may separate from solutions as liquid drops [591].

If coacervates are considered to be an intermediate state between a solution and a colloidal precipitate, it is apparent that during the precipitation of proteins from solutions by neutral salts, coacervates can form at one of the stages. In many cases this process is accompanied not only by a dehydration of the protein particles but also by the removal of charge from them. The formation of a coacervate also depends on the chemical nature of the salt employed [548].

Salts affecting the formation of coacervates are: 1) salts forming coacervates: Li_2SO_4, Na_2SO_4, Na_2SO_3, $Na_2S_2O_3$, $(NH_4)_2 \cdot SO_4$, K_2CO_3, $FeSO_4$, $MgSO_4$, $NiSO_4$, NaCl, NH_4Cl, $NaNO_3$, $ZnSO_4$, sodium acetate, sodium lactate, sodium citrate; 2) salts which do not form coacervates: LiCl, Na_2CO_3, $BaCl_2$, $CaCl_2$, $AgNO_3$, $Pb(NO_3)_2$, $Ur(NO_3)_2$, [sic] $NaHCO_3$, $NaBO_3$, Na benzoate, $Mg(NO_3)_2$, Na salicylate, Na butyrate, sodium glycerophosphate, $NaNH_4$ molybdate, KNO_3.

Both the chemical nature of the salts and the chemical composition of the organic compounds considerably influence the preparation of coacervates.

Compounds forming coacervates	Compounds not forming coacervates	Compounds forming coacervates	Compounds not forming coacervates
Methanol	Glycol	Resorcinol	—
Ethanol	Glycerin	Ethyl acetate	Urea
Propanol	Mannitol	Chloral hydrate	Formaldehyde
Pentanol	—	Ethylurethan	Acetaldehyde
Phenol	—	Dioxane	

Simple coacervates may sometimes form in certain parts of the cell. As a rule, however, in protoplasm the proteins are combined with the most diverse substances in the form of complex proteins called proteides. For this reason, complex coacervates are of much greater interest for biology.

Complex coacervates. Coacervates of this type can be obtained from oppositely charged solutions. When complex coacervates are formed, there takes place not only a decrease of the forces of hydration as a result of the neutralization of charges, but also an increase in the forces of attraction between positively and negatively charged particles of the dissolved substance [506, 552, 579]. In most cases, the following combinations are used for the preparation of such coacervates: protein-carbohydrate, protein-protein, protein-nucleic acid, carbohydrate-nucleic acid, etc. In addition, the existence of multicomponent coacervates from various compounds is also possible. /29

Complex coacervates are formed in a pH range bounded by the isoelectric points of the substances comprising them.

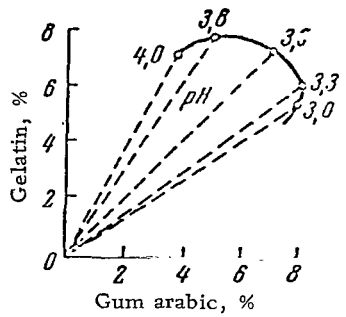


Figure 13. Formation of Coacervates at Various pH Values as a Function of the Ratio of Protein and Carbohydrate Solutions. Points on Curves - pH Values at which Coacervates are Formed.

It is most convenient to study the properties of complex coacervates by using those consisting of gum arabic and gelatin as an example [490, 546, 553, 575, 905]. The isoelectric point of acid gelatin lies at pH 4.82–5.0, and that of gum arabic at pH 1.23. The coacervate can be obtained at a slightly more acidic pH then the isoelectric point of gelatin and at a more alkaline pH than the isoelectric point of gum arabic. In this pH range, gelatin is charged positively and gum arabic negatively. As the pH decreases, the quantity of positive charges on gelatin increases, and correspondingly more gum arabic is needed for their neutralization and for the coacervate to form. Conversely, an increase of the pH causes an increase in the negative charges of gum arabic, and therefore the formation of the coacervate requires the addition of a large amount of gelatin. If instead of the latter, other acid proteins with an isoelectric point close to gelatin are taken a similar situation will arise. The relationship between the pH and the ratio of concentration of gum arabic to gelatin is shown in Fig. 13.

If 0.67% solutions of gelatin and gum arabic are combined in ratios of 4:6, 5:3, 9:1, etc., the pH of such coacervates will also change correspondingly [546, 566]. The highest concentration of gelatin and gum arabic solutions at which coacervates can still be obtained is 6%, and the lowest, 0.0016%. When different concentrations of gelatin and gum arabic are employed, both gum arabic and gelatin will always remain in the equilibrium liquid. Under optimum conditions, up to 84% of the molecules are contained in the coacervate layer or drops, and 16% remain in the equilibrium liquid [509, 550, 561—562].

Gelatin may form coacervates not only with gum arabic, but with other carbohydrates as well, for example, with sodium arabinate, agar and also various starches [585, 841—845]. Gelatin molecules have a greater ability to attract water than starch, since gelatin is richer in hydrophilic groups than starch. Gelatin /30
is capable of removing water from dissolved starch and even precipitating it. In 1898, Buchli observed the formation of drops by combining solutions of gelatin and starch. For example, a coacervate obtained from a 5% solution of potato starch and a 2% solution of gelatin upon addition of 0.001 N HCl at 35° can exist for only 3 hours, after which the solutions begin to age and the coacervate breaks down. Rice, corn and wheat starches are less hydrated then potato strach. Solutions of rice or corn strach produce good but also unstable coacervates. The introduction of phosphoric acid into the starch molecule

increases its negative charge. Such amylophosphoric acid readily forms coacervates with gelatin. If amylopectin is used instead of amylophosphoric acid, it must be taken in larger amounts, since the charge of amylopectin is less than that of amylophosphoric acid [746,800].

In order to prepare coacervates with agar, solutions of 2% agar and 10% gelatin heated to 50° are employed. A distinguishing feature of coacervates of gelatin with agar and starches is their instability, and also the tendency to produce coacervate drops only from solutions with a high initial concentration of the substances.

With few exceptions, various acid and alkaline proteins form stable coacervates with gum arabic. The isoelectric points of acid proteins lie most frequently in the range of pH 5-6, and those of alkaline proteins, in the range of pH 7. For instance, the isoelectric point of histone isolated from nuclei of the calf thymus ranges from pH 9.5 to 10.0; gelatin called ichthyocolla has an isoelectric point of pH 9.0 [11,214,430,551,610,832]. The most alkaline protein, clupein, is a protamine obtainable from herring milt. Its isolectric point is at pH 12.0 [251,749].

For this reason, coacervate drops whose composition includes alkaline proteins can be obtained at a more alkaline pH than when acid proteins are employed.

Gum arabic is isolated from various types of acacias. The preparations differ slightly from one another in both chemical composition and properties. Depending on the type of gum arabic, one can obtain coacervates with hemoglobin in the pH range from 2.2 to 4.0 at 50°[374,655]. Gum arabic gives coacervates with serum albumin [129,325,593,782].

The coacervate from histone and gum arabic exists at pH 6.0. During formation of the coacervate from equal amounts of alkaline proteins (protamines) and carbohydrates, the pH of the coacervate is 7.0. A change of the quantitative proportions causes a shift of the pH [551]. For example, with clupein or with its /31
salt clupein sulfate, gum arabic gives various coacervate systems beginning at pH 5.0 to 7.0; the more gum arabic is present, the more acidic is the pH of the coacervate.

If solutions of two different proteins (acidic and alkaline) at the same pH value carry opposite charges, complex coacervates can be obtained by combining them. The greater the difference between the isoelectric points of the proteins, the more readily do they form coacervates. Well-formed drops can be obtained by combining histone with gelatin, histone with serum albumin, clupein with gelatin, clupein with egg albumin, sickle protamine with gelatin, etc. In most cases, such coacervates are formed in the pH range from 5 to 8. The pH value

depends on the ratio of the components: the more alkaline protein is present, the more alkaline is the pH of the coacervate [129,708].

Of major importance for the development of evolutionary biochemistry is the study of coacervates made up of synthetic nonspecific polymers, particularly peptides and polynucleotides, which may have been synthesized abiogenically on the earth [8,779,821,885,888]. Such coacervates were first obtained in 1963-1964 by Oparin, Serebrovskaya and others. Coacervates consisting of RNA + polylysine, histone + polyadenylic acid and polylysine polyadenylic acid produced well-formed drops. Thus, the formation of coacervates is possible in the course of synthesis of polymeric compounds on earth with the participation of nonspecific polymers [268].

A very intersting property of alkaline proteins is that they readily form coacervates with nucleic acids. This is explained by the fact that the latter have an isoelectric point in the acid range of pH 1.2 to 1.6 [723,902].

Coacervates from nucleic acids and histones form in the pH range of 6.0-8.0, and those with the participation of protamines have a higher alkaline pH. Such coacervates may be of major interest, since complexes from nucleic acids and alkaline proteins are found in cells, nuclei and ribosones, and in the latter, the protein and nucleic acid can be separated from each other [427,498,617,674, 709,909].

Nucleic acids can also produce coacervates with acid proteins, for example, with serum albumin in the 3.8-4.5 pH range [262-441-900].

A coacervate made up of gum arabic, gelatin and yeast nucleic acid has been studied fairly thoroughly. In this case, the nucleic acid may coacervate with gelatin and also form a compound with the coacervate already formed from gelatin and gum arabic, producing, a three-component coacervate. In such a coacervate, nucleic acid and gum arabic are charged negatively, and gelatin is positively. The existence of different types of coacervates with nucleic acids /32
depends on the pH: at pH 3.36, the coacervate consists mainly of gum arabic and gelatin; at pH 3.48, of gelatin, gum arabic and nucleic acid; at pH 3.8, of gelatin and nucleic acid [589—590, 592].

There are many cases where the drops of coacervates consisting of gum arabic and gelatin contain inclusions of droplets of coacervate from gelatin and nucleic acid, which is observed upon dying with methyl green and also from the absorption of UV light in the wavelength range of 260-270 mμ [116, 138, 554].

In protein-nucleic coacervates, the protein and nuclein acid most probably form a nucleoprotein [43]. However, there exists a hypothesis according to which nucleic acid is adsorbed on particles of the protein [508], which is less likely

Special coacervates are obtained by using dyes. Many dyes are employed in cytology for observing cellular structures. For this reason, coacervates from dyes are also of a certain interest to biology.

The dyes themselves can become adsorbed on high molecular compounds to give coacervates. In preparing such coacervates is the magnitude and sign of the charge of both the solution and the dye, are of major importance [511].

For example, if solutions of acid proteins are charged negatively, they readily produce coacervates with basic dyes charged positively.

As the temperature is raised, the formation of a coacervate requires a larger quantity of dye, since the adsorption of the dye by the protein increases.

Simple and complex coacervates are readily dyed by various dyes [511, 513, 736]. The capacity to be dyed may be considered as one of the properties of the coacervate. However, during the dying, a new coacervate is formed in addition to the existing one. For instance, droplets consisting of gum arabic, gelatin and methylene blue make up such a coacervate. Upon addition of a solution of methylene blue to coacervate drops obtained from gum arabic and gelatin, the droplets absorb (adsorb) the dye and acquire a blue color. A study of a series of dyes has shown that far from all the dyes selectively color coacervate drops only. There are many cases where both the drops and the surrounding equilibrium liquid are dyed. The best results for protein-carbohydrate coacervates are obtained with dyes producing a so-called lifetime color with the protoplasm. /33
Such dyes include methylene blue, neutral red, eosin, etc. It turns out that the state of the protein is also of great importance for the formation of coacervates with dyes.

Denatured proteins coacervate poorly with dyes.

It is known that the protein molecule may have a primary, secondary, tertiary, and quaternary structure, whose characteristics can be found in many studies [138, 471, 735, 854,857, 858].

The denaturization of a protein consists in the destruction of its secondary structure, due to the rupture of hydrogen bonds under the influence of various factors. Urea is a typical agent breaking hydrogen bonds, and there exists a definite relationship between the urea concentration and the quantity of broken hydrogen bonds. After proteins have been treated with urea, they no longer give coacervates with dyes.

Table 4 shows data on the influence of temperature and irradiation with UV light on the coacervation of proteins with the participation of dyes [384—386].

The action of these physical factors frequently causes more profound changes affecting not only the secondary but also the primary structure of proteins [17, 281].

TABLE 4. Effect of Denaturization of Proteins on Coacervation With Dyes (after Feldmann)

Specimen	pH	Temperature*, °C					Time of irradiation with ultraviolet light, hours			
		50	55	60	65	70	1	2	1	2
		Neutral red								
Horse serum albumin	6.5	+	+	+	—	—	At 0°		At 20°	
	7.0	+	+	+	—	—	—	—	—	—
	7.4	+	+	+	+	+	+	+	—	—
	8.0	+	+	+	+	+	—	—	—	—
Horse blood serum	—	+	—	—	—	—	—	—	—	—
Frog blood serum	—	+	+	—	—	—	—	—	—	—
		Nile blue								
Egg albumin	6.5—8.0	+	+	+	+	—	—	—	—	—

*At the indicated temperatures, 0.1% protein solutions were heated for 10 min; plus sign - formation of coacervate; minus sign - no coacervates are formed.

In this case, both individual proteins and mixtures of different proteins (for example, proteins of blood serum) [385] lose their ability to form coacervates with dyes. Denatured proteins coacervate poorly not only with dyes but with other compounds as well. For instance, coacervate from serum albumin, gum and clupein breaks down as a result of the denaturization of the protein. /34

Bank contends that coacervates are formed with dyes when both animal and plant tissues are dyed [439, 440]. The separation of vacuoles within the cells during dying results from the formation of coacervates. For instance, when toluidine blue is added to a gum arabic solution, at the point of contact between the carbohydate and the dye a drop is formed in which a pulsating vacuole arises which alternately increases and decreases in size [511].

Coacervates are formed when salts are added to many colloidal solutions of dyes. Trypaflavine produces well-formed coacervate drops with neutral salts; this coacervate dissolves on heating.

With sodium citrate, a coacervate is formed at 16—18°. A further increase in temperature causes the drops to dissolve, and in the case of potassium metasulfate, the formation of a coacervate takes place on heating [537, 591—592].

Different coacervates can be obtained from solutions of proteins, carbohydrates, phosphatides, and sols of fatty acids with the participation of mineral salts, particularly cobalt hexonyl nitrate [548 554, 577, 584, 614]. A change in the concentration of the added salt may cause a charge reversal of the entire coacervate. The coacervates are obtained by heating solutions of glycogen, soluble starch, and gum arabic with such salts as $Th(NO_3)_4$, $AlCl_3$, $PtCl_3$. On cooling, these coacervates break down. Solutions of gelatin and sodium arabinate with chlorides of lead, lanthanum, copper, manganese, cobalt, nickel and strontium and also sodium chondroitinsulfuric acid and salts of nucleic acids with $PtCl_3$ and $Pt(NO_3)_3$ may form coacervates of this type. An oily coacervate is obtained from gelatin and sodium alkylsulfates [592,798, 840].

In the case at hand, adsorption of the alkylsulfate on ketoimide groups of gelatin is postulated. The plasma contains a great variety of salts as well as protein, carbohydrate and lipid compounds for the formation of such coacervates.

Coacervates can be obtained by adding inorganic salts (KCl, $CaCl_2$, etc.) to solutions of lipids. When lipid coacervates are formed, the forces of attraction between the colloidal micelles increase owing to the hydrocarbon radicals which enter into the composition of lipids. Lipid coacervates can be obtained from /35
phosphatides, fats fatty acids and their derivatives [526--529, 544, 545, 558, 580, 582, 588, 613].

Phosphatides are amphoteric compounds. For this reason, they form coacervates with cations as well as anions and ampholytes. Not only mineral salts, but also carbohydates, proteins and other substances can add to phosphatides. In such cases, a mixed type of coacervates is formed. An example of this type is the system formed by sodium arabinate, lecithin and lanthanum nitrate; salts of lead or uranium oxide can be taken instead of lanthanum. Sodium arabinate with lecithin is a complex coacervate on which lanthanum salts are adsorbed. Coacervates from soy bean lecithin have been studied in most detail [612]. Lecithin forms coacervates with carbohydates and with nucleic acids. It is charged positively in such systems, while nucleic acid and the carbohydate are charged negatively.

Coacervates consisting of lecithin and various proteins such as casein, egg albumin, glycinine, clupein, etc. have been throughly studied [609, 531, 722]. The lecithin-protein coacervates exist in the pH range between the isoelectric points of the protein and lipoid (e.g. lecithin). A coacervate from acid gelatin and lecithin can be obtained at pH from 2.7 to 4.82. In this reaction of the solution, gelatin is charged positively and lecithin negatively. The lecithin-protein coacervate at 20° is in the form of viscous drops, and vacuoles are formed in the latter on cooling [611, 527].

A negative temperature coefficient is characteristic of coacervates obtained from fats and fatty acids: the higher the temperature, the smaller the volume

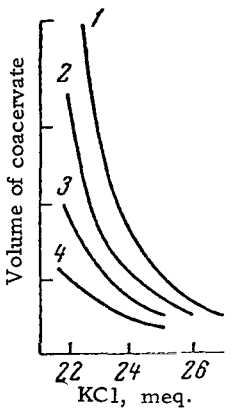


Figure 14. Change in the Volume of Coacervate Layer (oleate - KCl) at Various Temperatures.

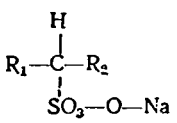
1—8°: 2—18,5°: 3—23°: 4—35°

occupied by the coacervate layer [597, 606]. Figure 14 shows the relationship between the volumes occupied by the coacervate layer and the temperature in a coacervate obtained from sodium oleate + KCl in water. The size of the layer also depends on the amount of salt of fatty acid taken. If the concentration of potassium laurate is 0.03 M, the coacervate layer is twice as large as when the concentration of the salt is 0.005 M [652].

Hemoglobin, albumin and pseudoglobin of the blood coacervate with myristylcholine present in a very low concentration (or the order of 0.015%) [523, 587, 606]. /36

Sodium oleate gives coacervates with such proteins as egg albumin, serum albumin, and also with globulins, etc. These proteins participate in the formation of coacervates with alkylsulfates.

Coacervates obtainable from alkysulfate derivatives have been thoroughly studied. The general formula of alkylsulfate derivatives is



where R_1 and R_2 are hydrocarbon radicals in which the number of carbon atoms ranges from 8 to 18. As in phosphatides, the alkylsulfate compounds contain hydrocarbon radicals and acid groups. The coacervates obtained from them are similar in properties to lipid coacervates [476, 477, 482]. For example, the coacervate from gelatin and an alkysulfate is oily and exists at pH 4.82. All lipid coacervates readily from films. For this reason, they are attributed a great significance in the formation of surface layers and membranes in cells [110—112, 546, 580—582].

The composition of multicomponent coacervates includes proteins, carbohydrates, lipoids, nucleic acids and various salts. Usually in such coacervates, the lipoids are charged positively, and nucleic acids and carbohydrates, negatively.

The above discussed coacervate from gum-gelatin and nucleic acid is a multicomponent coacervate. If histone is added to it, a four-component coacervate, gum-gelatin-RNA-histone, is obtained. Liebl et al. [211, 772, 773] obtained even

TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

Number	Composition	Reference
	Protein system	
1	Amandin - cooled H_2O	[840]
2	Gelatin - Na_2SO_4	[841, 845]
3	Gelatin - Hexol nitrate	[557]
4	Gelatin - C_2H_5OH	[557]
5	Gelatin - resorcinol	[557]
6	Chloroamines - C_2H_5OH	[597]
7	Clupein sulfate + alkali	[748]
8	Salmine - alkaline	[748]
9	Sturine - alkaline + alcohol	[748]
10	Proteins - phenols	[504]
11	Proteins - mineral salts	[597]
12	Gelatin - sulfosalicylic acid	[845]
13	Edestin - NaCl	[720]
14	Gelatin (ichthyocolla)	[610]
15	Gelatin - KCNS	[940]
	Carbohydrate Systems	
16	Glycogen + $AlCl_3$, $Sn(NO_3)_4$, $PtCl_3$	[574]
17	Starch - $AlCl_3$, $Sn(NO_3)_4$, - $PtCl_3$: hexol nitrate, acetone	[574]
18	Gum arabic - C_2H_5OH	[757]
19	Agar - hexol nitrate	[605]
20	Gum arabic - Na_2SO_4 - acetone	[564]
21	Gum arabic - Na_2SO_4 - alcohol	[757]
	Coacervates with Tannin	
22	Tannin - theobromine	[901, 902]
23	Tannin - agar	[503]
24	Tannin - glycogen	[503]
25	Tannin - starch	[503]
26	Tannin - casein	[503]
27	Tannin - gelatin	[510]
28	Tannin - gelatin - acetone	[501, 502]
29	Gallic acid - gelatin	[627]
30	Digallic acid - gelatin	[627]
	Protein-Carbohydrate Systems	
31	Serum albumin -gum arabic	[593]
32	Hemoglobin - gum arabic	[655, 656]
33	Gelatin - Na arabinate - $MgCl_2$ - mineral salts	[597]
34	Gelatin - gum arabic	[597]
35	Gelatin - agar	[597]
36	Gelatin - carragheen	[843]

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TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

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Number	Composition	Reference
37	Gelatin - potato starch	[845]
38	Gelatin - rice starch	[843]
39	Gelatin - wheat starch	[843]
40	Gelatin - phosphorylated starch	[746]
41	Gelatin - gum arabic	[610]
42	Leucosine - phosphorylated starch	[746]
43	Potato albumin - phosphorylated starch	[746]
44	Gelatin - amylopectin	[746]
45	Clupein - sulfate - gum arabic	[566]
46	Sickle protamine sulfate - gum arabic (at various pH's)	[141]
47	Starred sturgeon protamine sulfate - gum arabic	[141]
48	Histone hydrochloride - gum arabic	[772, 141]
49	Gelatin - gum arabic - Cu	[772]
50	Same	[129]
51	Gelatin - chondroitinsulfate	[755]
52	Gelatin chondroitinsulfuric acid - salts of Mn, Co, Ni, St.	[508]
53	Gelatin - saponin	[880]
	Protein - protein	
54	Human serum albumin - histone (at various pH values).	[772, 129]
55	Histone - gelatin	[129]
56	Clupein - ichthyocolla	[551]
57	Clupein - casein	[551]
58	Clupein - serum and egg albumins	[566]
59	Clupein - gelatin	[566]
60	Sickle protamine - gelatin	[129]
61	Starred sturgeon protamine - gelatin	[141]
62	Pancreatin - gelatin - Na_2SO_4	[627]
	Coacervates with Nucleic Acids	
63	RNA with salts of Pt, Ca, etc.	[583—584]
64	RNA Na - cobalt hexol nitrate	[583—584]
65	RNA - alkaloids	[194]
66	RNA - polylysine	[268]
67	RNA - serum albumin	[329]
68	RNA - gelatin	[551]
69	RNA - ichthyocolla	[551]
70	RNA - hemoglobin	[655—656]
71	RNA - histone at various pH's	[773, 901]
72	RNA - histone $MgCl_2$	[210, 211]
73	RNA - clupein sulfate (at various pH's)	[129, 551]
74	RNA - sickle protamine	[141]
75	RNA - starred sturgeon protamine	[129]

TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

Number	Composition	Reference
76	RNA - gelatin - gum	[508]
77	RNA - gelatin - gum - salts of Ca, Mg, Ba	[590]
78	RNA - gum - myristylcholine	[654—656]
79	RNA - trypsin and several other proteins	[193, 194]
80	RNA - serum albumin - gum arabic	[262]
81	Na DNA with salts of Pt, Ca etc.	[583—584]
82	Na DNA gelatin	[551]
83	Na DNA ichthyocolla	[551]
84	Na DNA clupein	[141]
85	DNA - histone at various pH's	[129, 773, 901]
86	DNA - starred sturgeon protamine	[141]
87	DNA - sickle protamine	[141]
88	Polyadenylic acid - histone	[272]
89	Polyadenylic acid - polylysine	[268]
	Coacervates with Purine and Pyrimidine Bases	
90	Adenine, cytosine, thymine, hypoxanthine, guanine, uracil-gelatin - gum arabic	[129]
	Coacervates with Mononucleotides	
91	Adenosine monophosphate - gum arabic - gelatin	[773]
92	Adenylic, guanylic, cytidylic, uridylic, thymidylic acids - gelatin - gum arabic	[129]
93	Coacervates with Amino Acids	
93	Gelatin - gum arabic + mixture of amino acids (alanine + phenylalanine + aspartic acid + arginine + histidine + lysine + cysteine) + glutathione	[210, 773]
94	Tyrosine - gelatin - gum arabic	[146]
95	Tyrosine - clupein - gelatin	[146]
96	Tyrosine - lgeatin - K oleate	[146]
97	Tryptophan - gelatin - gum arabic	[146]
98	Tryptophan - clupein - gelatin	[146]
	Coacervates with Dyes	
99	Trypaflavine, trypaflavine with salts	[591]
100	Gum arabic - methlene blue, trypaflavine	[579]
101	Gum arabic - toluidine blue	[505]
102	Gum arabic: Nile blue, brilliant, cresyl blue, neutral red, crystal violet	[385]
103	Gelatin, eosin, tropeolin, neutral red	[579]
104	Gelatin - Na	[579]
105	Gelatin: Nile blue, trypan red, aniline orange, indigo carmine, methyl orange	[386]
106	Gelatin - fast green	[736]
107	Egg albumin: Nile blue, bright cresyl blue, neutral red, methylene blue, crystal violet, toluidine blue	[385, 386]

TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

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Number	Composition	Reference
108	Serum albumin: Nile blue, bright cresyl blue, netural red, toluidine blue, methylene blue, crystal violet ...	[383—385]
109	Histone - fast green	[736]
110	Na salt of RNA: Nile blue, crystal violet	[736]
111	Na salt of RNA - methyl green	[579]
112	Gelatin - gum arabic, RNA - methyl green	[554]
113	Gelatin - gum arabic: methylene blue, neutral red	[524]
114	Gelatin - gum arabic toluidine blue	[537]
115	Gelatin - gum arabic - nile blue	[386]
116	Histone - gelatin ..... Histone - gum arabic Histone - serum albumin Serum albumin - gum arabic Clupein - gum arabic + various dyes	[134]
117	Sickle protamine sulfate - gum arabic - eosin	[134]
118	Starred sturgeon protamine sulfate - gum arabic toluidine blue	[134]
119	Histone - serum albumin - dichlorophenolindophenol ...	[329]
120	Na oleate gelatin - tropeolin	[579]
121	Gelatin - lecithin - methylene red	[135]
122	Serum albumin - gum - RNA - toluidine blue, methyl green pyronine	[262]
123	Histone - gum arabic - glucose - 1- phosphate - NaF, starch - carmine, toluidine blue	[134]
124	Alkyl amide derivatives + various dyes	[224]
	Coacervates with Fluoroscent Dyes: Acridine Orange, Euchrysine and Auerophosphine	
125	Sickle protamine sulfate - gum arabic	[129]
126	Starred sturgeon propylene sulfate - gum arabic	[129]
127	Clupein sulfate - gum arabic	[129]
128	Serum albumin - gum arabic	[129]
129	Gelatin - gum arabic	[129]
130	Sickle protamine sulfate - gelatin	[129]
131	Clupein - gelatin	[129]
	Phosphatide Coacervates	
132	Lecithin - heptane - butanol	[545]
133	Lecithin - octane - butanol	[545]
134	Lecithin - butanol - NaCl	[580]
135	Cephalin - butanol	[581]
136	Lecithin - decyl alcohol and other higher alcohols	[582]
137	Lecithin - oleyl alcohols	[544]

TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

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Number	Composition	Reference
138	Lecithin – lauryl alcohol	[544]
139	Lecithin – cetylmethylammonium bromide (NaCl)	[527, 528]
140	Lecithin – Na salicylate	[527—528]
141	Lecithin – $CaCl_2$ chloral hydrate	[527—528]
142	Lecithin – gum arabic	[540]
143	Lecithin – nucleic acid	[541]
	Protein – Phosphatide – Protein	
144	Egg albumin – lecithin	[481]
145	Glycinin – lecithin	[612]
146	Gelatin – lecithin	[577]
147	Ichthyocolla – lecithin	[612]
148	Casein – lecithin	[612]
149	Histone – lecithin	[450]
150	Clupein sulfate – lecithin	[612]
	Oleate Coacervates	
151	K-oleate – H_2O – KCl	[598]
152	Na-oleate – H_2O – KCl	[604]
153	K-laurate – H_2O – KCl	[653]
	Protein – Oleate Coacervates	
154	Serum albumin – K oleate	[653, 654, 656]
155	Egg albumin – K oleate	[654—656]
156	Egg albumin – Na oleate	[654—656]
157	Gelatin – K oleate – KCl	[606]
158	Gelatin (ichthyocolla) – K-oleate – KCl	[487]
159	K oleate – tetraborate – alkylsulfate	[596]
160	Gelatin – alkylsulfate with chain from C_8 to C_{18} carbon atoms	[849]
161	Blood serum albumin–myristylcholine	[656]
162	Hemoglobin – myristylcholine	[655—656]
163	Blood pseudoglobulin – myristylcholine	[655—656]
164	Gelatin – gum tetrahydroraphthalene	[522]
165	Gelatin – cetyltrimethylammonium bromide – KCNS	[602]
166	Cholesterol – gelatin	[602]
167	Lecithin – cholesterol – $CaCl_2$	[602]
168	Gelatin – gum arabic – starch	[148]
169	Gelatin – agar – sucrose	[457]
170	Histone – glucose – 1 – phosphate – gum	[129]
171	Sickle protamine sulfate – gum arabic – glucose – 1 – phosphate	[129]
172	Gelatin – gum arabic – albumin	[773]
173	Gelatin – gum arabic – hemoglobin	[773]
	Multicomponent Coacervates	
174	Histone – gelatin – gum arabic – RNA	[773]

TABLE 5. Chemical Composition of Hydrophilic Coacervate Systems

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Number	Composition	Reference
175	Histone - gelatin - gum arabic - RNA - Cu	[773]
176	Gum arabic (Na arabinate) - gelatin - histone - RNA + mixture of nine amino acids (cysteine, aspartic acid, glutamic acid, alanine, valine, phenylalanine, histidine, lysine, arginine)	[211]
177	Adenosine monophosphate - Na arabinate - gelatin - histone - RNA	[211]
178	Hemoblobin - Na arabinate - gelatin - histone - RNA ...	[211]
179	Myoglobin - Na arabinate - histone - RNA	[211]
180	Serum albumin - Na arabinate - gelatin - histone - RNA.	[211]
181	Lecithin - cholesterol - fat - $CaCl_2$	[602]
182	Serum albumin - K-oleate - chlorophyll - alcohol	[332]
183	Serum albumin - K-oleate - RNA - chlorophyll	[336]
	Coacervates from Biological Liquids	
184	Horse blood serum - methylene blue	[386]
185	Frog blood plasma - methylene blue	[386]
186	Hemolymph of pupae of mulberry silkworm	[129,346,347]
187	Hemolymph of pupae of oak silkworm - gum arabic	[129]
188	Human blood serum - gum arabic	[822]
189	Blood serum + gum arabic - sun flower oil + sudan red .	[223]
190	Yeast juice + gum arabic	[129]
191	Sea urchin protoplasm + $CaCl_2$	[696]
192	Liver cell homogenates	[433]
193	Serum albumin, globulin, egg albumin, glycogen + NaCl, $CaCl_2$, $MgCl_2$	[827]
194	Aloe juice, pH <7	[904]
195*	Protoplasm of plant and animal cells	[249]

*The remaining 17 systems containing the enzymes are given in Chapter 7.

more complex systems with this coacervate. In addition to the indicated compounds, the coacervate contained a mixture of nine amino acids, or hemoglobin, myoglobin, serum albumin, and adenosine-3'-monophosphate.

Another example of a multicomponent lipoid coacervate is one whose composition includes lecithin-sterols-fat + H_2O and $CaCl_2$.

An interesting aqueous coacervate was obtained by Serebrovskaya et al. from chlorophyll-RNA-serum albumin-K oleate. Usually, chlorophyll dissolves in organic solvents, but not in water. In a mixture with RNA, chlorophyll acquired the ability to dissolve in water. The spectrum of such a complex is found to be

similar to the spectrum of chlorophyll in a live green leaf. In this system, chlorophyll had a stronger fluoroscence than in solution [332—336].

The preparation of multicomponent coacervate systems was begun chiefly in the last few years in order to study enzyme reactions. These systems are analyzed in detail in Chapter 6.

In addition to coacervates consisting of pure chemical compounds, well-formed coacervate drops are produced from protoplasm [358, 696, 697] and biological liquids rich in proteins. Such liquids include blood plasma and serum, hemolymph of insect pupae, and various homogenates and lysates of cells [178, 208—209, 343, 345, 347, 775—776, 903, 904]. These liquids readily coacervate either by themselves or upon the addition of other compounds, for example, gum arabic. Anderson [433] separated precipitates from liver cell homogenates by centrifuging. The transparent solution was subjected to dialysis in sucrose and a phosphate buffer at pH 7.5. The liquid was then allowed to stand in the cold; the pH thereupon changed to 5-6, and various coacervate drops or bubbles and other structures were formed. /43

Thus, hydrophilic coacervates can be obtained from compounds most diverse in chemical composition in various pH ranges (approximately from 2 to 11) and at temperatures of 8 to 70°.

A list of hydrophilic coacervate systems is given in Table 5.

From the data of Table 5 it follows that coacervates are highly diversified in chemical composition.

Models involving the participation of gelatin and gum arabic are encountered most frequently in these coacervates. Although gelatin is far removed from natural native proteins, the two-component coacervate system consisting of gelatin and gum arabic is a kind of experimental "frog" on which the fundamental properties of coacervates can be clearly demonstrated.

Quantitative and qualitative changes in coacervates can arise under the influence of various substances. Of greatest importance are salts and low molecular organic compounds.

Chapter 3

EFFECT OF VARIOUS COMPOUNDS ON THE PROPERTIES AND STRUCTURE COACERVATES /44

Different chemical compounds and physical factors (temperature, pressure, electric current, etc.) change the volume and structure of coacervate layers, and the size, shape and structure of coacervate drops. By using these factors one can orient their action in the desired direction.

Effect of Electrolytes and Nonelectrolytes

Mineral salts are an indispensable component part of protoplasm. As was indicated above, many coacervate systems are formed only in the presence of salts. The latter have a definite effect on already existing coacervates, since being themselves carriers of charges, they are capable of dehydrating substances and changing the charge of the coacervate.

In addition, salts have a specific effect on coacervates owing to a different chemical composition.

Salts as Dehydrating Agents. If the added salt has a greater affinity for water than the coacervate, it removes the water from the coacervate, dehydrates it, breaks it down, and converts it into a precipitate. The more hydrated the coacervate, the harder it is for it to hold water and the less salt is required for its precipitation[508, 521]. For example, the coacervate from gum arabic and gelatin precipitates faster, since it is more hydrated than the coacervate from gelatin and DNA.

Salts as Electrically Charged Compounds. Since mineral salts are electrolytes, they decrease the charge of the coacervate or even eliminate it completely, which in the end causes the coacervate to precipitate. Salts can also increase the charge of the coacervate, thus increasing its stability.

In some cases, salts cause a change of charge on the entire coacervate. /45
For example, this change takes place upon addition of various amounts of KCl to the coacervate obtained from a 1% solution of sodium nucleate and 2% gelatin.

Upon adding 10 meq of KCl, the entire coacervate becomes negatively charged, and when 100 meq of KCl is introduced, it becomes positively charged. The lowest stability of the coacervates is observed at the isoelectric point of the hydrophilic compound from which the coacervate is obtained. The coacervate from lecithin and gelatin breaks down most easily at pH 2.7 (the isoelectric point of lecithin) and at pH 4.82 (the isoelectric point of gelatin) [508, 600, 521, 929].

Positively charged coacervates are most sensitive to neutral salts. For example, the coacervate from clupein sulfate and lecithin at pH 7.39 breaks down even in the presence of 0.06 meq of $CaCl_2$. Occasionally, a slight change of the chemical nature of one of the components of the coacervate changes its stability. Thus, the coacervate from gelatin and soybean lecithin is more stable in the presence of neutral salts than the coacervate obtained from the same gelatin and egg lecithin [531, 532, 558]. The higher the valence of the salt, the stronger its influence on the coacervate. Of great importance is the chemical composition of the coacervate and of the added salt. Table 6 shows data on the precipitating effect of salts on coacervates.

TABLE 6. Precipitation of Coacervates with Salts (in meq)

Coacervate composition	pH	NaCl	KCl	$CaCl_2$
Clupein and soybean lecithin	7.44—7.39	170	675	130
Gelatin and egg lecithin	3.44	16	18	2.5

The data of Table 6 indicate that in order to achieve the same effect, different amounts of different salts are required, and in the case under consideration, the specificity of the action depends on the nature of the cation, since the anion is always the same.

In the strength of the destructive action on coacervates, the anions and cations of the salts can be arranged in the following sequences:

Sequence of cations: Li>Na; Ca>Mg>Sr>Ba.

Sequence of anions: $KCl < \frac{K_2SO_4}{2} < \frac{K_3[Fe(CN)_6]}{3} < \frac{K_4[Fe(CN)_6]}{4}$.

If univalent salts with the same cations are present, their action depends on the chemical nature of the anion.

The depressing action of anions is represented in the form of the following sequence:

$$KCl < KNO_3 < KI < KCNS < \frac{K_2SO_4}{2}.$$

Such salts as $CaCl_2$ have an antagonistic effect on coacervates. The antagonism of these salts shows up with particular clarity upon their addition to the coacervate from lecithin and cholesterol. As the $CaCl_2$ concentration rises, the amount of added NaCl should also be increased in order to avoid substantial changes in the coacervate. A similar situation is observed upon absorption of $CaCl_2$ and NaCl by cells. Other antagonist ions are $(Th(NO_3)_4$ and $NaNO_3, UO_2(NO_3)_2$ and $NaNO_3$, the pair $\frac{UO_2(NO_3)_2}{NaNO_3}$ acting more strongly then $\frac{Th(NO_3)_4}{NaNO_3}$. /46

The electromotive force arising between the salt solution and the equilibrium liquid and also between the coacervate layer and the salt solution has been measured. Troshin showed that the emf for the liquid-salt system changes with the chemical composition of the salt cation in the following order: $Cs>Rb>K>>NH_4>Na>Li$ and is in agreement with the lyotropic series.

The emf for the system coacervate layer — salt changes in a different manner: $K>Rb>NH_4>Cs>Na>Li$. This sequence of salt cations is characteristic of protoplasm [375—377].

It is still unclear when the ions of salts in molecules of proteins, carbohydrates, nucleic acids, and other compounds are located in coacervates.

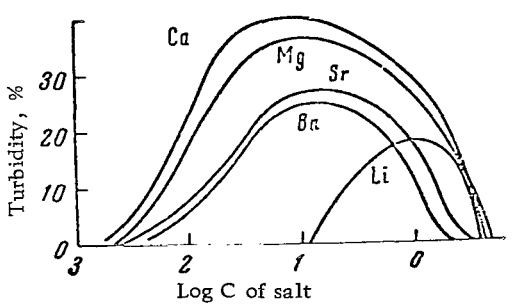


Figure 15. Effect of Salts on the Formation of Coacervate from Lecithin and Carregheen (carbohydrate from moss). The Percentage Turbidity Corresponds to the Amount of Coacervate.

Salts can not only break down coacervate layers and drops, but also promote their enlargement (Fig. 15). Under the influence of salts, the pH at which a given coacervate is formed can change. For example, in the absence of salts, the optimum of the formation of a coacervate from egg albumin and gelatin lies around pH 4.82, and upon addition of 20 meq of KCl, it shifts to pH 3.0.

An increase in the content of calcium chloride in the lecithin-sterol-fat coacervate causes the pH of the latter to shift to the alkaline side [508—509, 565, 600, 607]. Hence, by adding different amounts of salt, one can obtain coacervates at different pH's, which is particularly important for using coacervate systems as models. /47

The salt concentration may change not only the pH of the coacervate, but also the size of the coacervate drops [509, 517, 575].

Table 7 shows the influence of KCl on the size of coacervate drops of gelatin-gum arabic [586].

TABLE 7. Effect of KCl on the Size of Drops and pH of Coacervate from Gelatin - Gum Arabic

pH	Without KCl	In the presence of KCl, meq				
		40	70	100	110	140
	Diameter of drops*					
2.9	124—130	137	150	—	—	—
3,1	106—109	108	116	134	—	—
3.4	101—102	100	98	112	144	—
3.6	100	98	96	101	126	—
3,8	100	99	98	96	114	—
4.0	102	100	98	93	102	145
4,2	106—104	104	99	95	96	136
4,4	115—113	116	105	99	95	111
4,6	127—125	130	117	106	96	103
4,8	—	—	127	114	99	102
5,0	—	—	—	125	105	103
5,2	—	—	—	132	113	108
5.4	—	—	—	141	122	117
5.6	—	—	—	—	—	123
5,9	—	—	—	—	—	130

*Minus sign indicates that no coacervate is formed.

A low salt concentration causes a reversible change in the size of the drops. A high salt concentration leads at first to the appearance of flocs, and then dissolves the entire coacervate [487].

Of particular importance is the influence of salts on lipid coacervates.

Drops of lipid coacervates are characterized by a dense surface layer called film. Salts as hydrophilic substances can decrease or increase the distance between the hydrophilic groups in drops or coacervate layers. As this distance changes, there is a corresponding change in the permeability of the films. Small amounts of KCl added to oleate coacervates attract hydrophilic groups. There /48
takes place a decrease in the distance between these groups, and hence a decrease in the permeability of the lipid film to water and substances dissolved therein. Hard KCl concentrations loosen the coacervate, increasing the distance between the hydrophilic groups. In this case, the permeability increases [480], [488, 513]. The calcium ion has the same effect on the coacervates as the potassium ion.

Thus, in the presence of an electrolyte salt, a change takes place in both protein-carbohydrate and lipid coacervates.

The situation is somewhat different in the case of the action of nonelectrolytes on coacervates.

In most cases, protein-carbohydrate coacervates are not sensitive to the addition of nonelectrolytes, which usually penetrate inside the coacervate layer or drops and remain there in the form of isolated droplets. This is the behavior of benzene, toluene, and other aromatic hydrocarbons. An exception are such compounds as alcohols, which precipitate coacervates. The precipitating capacity of an alcohol increases with the number of carbon atoms it contains. For example, 7.7% ethanol has the same effect on coacervates as 6.4% propanol. The strongest precipitating property is displayed by alcohols with 14 carbon atoms in the molecule [563, 598-599, 663].

Nonelectrolytes have a strong effect mainly on liquid coacervates [-553].

Nonelectrolytes occupy positions between the carbon chains of liquid particles of the coacervate. If a nonelectrolyte attracts these chains to itself, the coacervate layer becomes denser and contracts, wheras if the chains are repelled, it swells and becomes diluted. This phenomenon has been studied by measuring the volume occupied by the coacervate layers before and after the addition of a nonelectrolyte [576, 581, 595].

In their ability to dilute or concentrate coacervates, nonelectrolytes can be subdivided into several groups according to their chemical composition.

Hydrocarbons. The greater the number of carbon atoms in a hydrocarbon, the stronger its concentrating influence on the coacervate. The following sequence applies to hydrocarbons with unbranched chains: pentanel < hexane < heptane < octane. Hydrocarbons with branched chains act more strongley than those with straight chains with the same number of carbon atoms [478, 486, 556, 576]. In their concentrating effect, aromatic hydrocarbons are arranged in the following order: benzene > naphthalene > phenathrene; phenathrenes < anthracenes. As their molecular weight increases, aromatic hydrocarbons loose their already low solubility and therefore possibly have a weak effect on coacervates. The /49
layers become diluted and swell upon addition of halo derivatives of hydrocarbons, since halides have hydrophilic properties. Most frequently, the introduction of halides into the molecule of a substance reinforces the effect it has on a coacervate. For example, benzoic acid increases the volume of an oleate layer of coacervate insignificantly. After a chlorine atom is added to this acid in the para position, the diluting effect of the acid increases markedly. An increase in the number of double bonds in hydrocarbons increases their concentrating effect on coacervate layers [544, 596, 599].

Alcohols. They contain the hydrophilic group OH. The longer the carbon chain of the alcohol, the weaker the manifestation of the diluting influence of the OH group [476,487,536,531,599].

The nature of the action of low molecular alcohols on oleate and phosphatide coacervates substantially depends on the pH of the coacervate. For example, at pH 9.3, butanol has a condensing capacity, and at a low pH it acts as a diluting agent.

The aromatic alcohol cholesterol concentrates the layers by occupying positions between the hydrocarbon radicals of the phosphatides and fatty acids [543,602].

In aldehydes, ketones and fatty acids, as the number of carbon atoms increases, so does the concentrating effect [483-484]. The condensing effect is pronounced in ethers with straight chains of carbon atoms but with branched ones. For example, diamyl ether concentrates oleate coacervate layers more than diisoamyl ether. Of major importance is the state of the fatty acids. If the fatty acid is in a dissociated form, it has only a slight influence on the size of the layer. In the case of undissociated molecule, however, its condensing effect on oleate coacervates is greater. The influence of esters is relatively difficult to study in oleate systems, since at pH 8 the esters hydrolyze to the acid and alcohol. As a rule, cyclic compounds including hydrocarbons and organic acids have a stronger condensing effect on coacervates than noncyclic ones. The degree of concentration of the coacervate layer upon addition of heterocyclic compounds depends on the noncarbon atom present in the ring. In strength of the condensing effect, these atoms are presented by the sequence $N<O<S<C$ [473, 474].

Amines. In the undissociated state, amines act like nonelectrolytes. Primary amines condense coacervate layers. If an OH group is introduced into amines, such derivatives for the most part dilute the coacervate layer [542]. Urethane and urea are strongly diluting agents, and their addition in small amounts causes the coacervates to dissolve.

Plant Growth Substances. Some of the above-discussed types of organic /50
compounds also include plant growth substances, which have specific effects on plants. For this reason, the influence of plant growth substances has been studied in greater detail.

According to the theory of Booij and Bungenberg de Jong [480], cell membranes contain the same protein-liquid complexes as do coacervates. Plant growth substances are located between the carbon chains in these groups. The carboxyl radicals of plant growth substances are oriented toward the protoplasm, i.e., toward the hydrophilic sol [488,543]. The location of the plant growth substances

in the membrane effects its permeability. In a parallel study of the effect of plant growth substances on plants and coacervates, it was found that a decrease in the physiological activity of plant growth substances in relation to plants coincided with a decrease of the permeability of oleate coacervates. A densification of the membrane took place in this case. Veldstra and other authors [941—944] checked about 30 different representatives of plant growth substances, chiefly derivatives of β-naphthylacetic acids.

As the number of double bonds decreases, the diluting effect of such compounds on coacervate layers decreases, and their physiological activity declines. However, the consistent behavior of plant growth substances with respect to coacervates and plant cells pertains only to physiologically active forms of plant growth substances. For example, the active form of the derivative of cinnamic acid is its cis configuration, but its trans form is not active.

Both cis and trans forms act on a coacervate. The trans form has a stronger influence than the cis form. Thus, a change in the volume of the coacervate layers can arise under the influence of both forms of plant growth substances.

Forms and Envelopes of Coacervate Drops

Various agents cause changes not only in the coacervate layer, but in the drops as well. Figure 16 and Table 8 demonstrate the action of vapors of a nonelectrolyte on lipid coacervates, and Table 9 shows the time of formation of deformed shapes of drops as a function of the chemical nature of the alcohol.

It turns out that the longer the carbon chain of the alcohol, the more active it is and the faster the deformed drops are produced (Table 8).

At the optimum temperature for the existence of a coacervate, the drops are largest in size. On cooling, they contract, forming flocs which frequently consist of fine droplets. If such flocs are carefully crushed with a cover glass, /51 the droplets change into rods. Further cooling causes the drops to disappear, and the flocs are converted into a dense precipitate [481, 487].

The formation of vacuoles in protein, nucleic and other drops under the influence of a direct current and distilled water is shown in Fig. 17, a, b.

Nonelectrolytes cause the formation of vacuoles in lipid and oleate coacervates.

Vacuoles are frequently formed after the nonelectrolyte vapor is driven off. /52 Obviously, the vacuolization does not occur immediately and is a response to previous actions. In the protoplasm of the organism, the same type of changes are observed [321].

TABLE 8. Effect of Nonelectrolytes on Lipid Coacervates

Character of changes in coacervate upon addition of nonelectrolyte vapors		Change in the choacervate after removal of nonelectrolyte vapors
Thickening effect on coacervate layers	Vacuolization: formation of large and small vacuoles and droplets. Granules in drops	Disappearance of vacuoles; Fine drops pass into solution Granules disappear, and myelin figures appear
Diluting effect on the coacervate layers	Total volume of the coacervate layer changes. Passing into solution	Vaculoization: fine drops, reformation of coacervates.

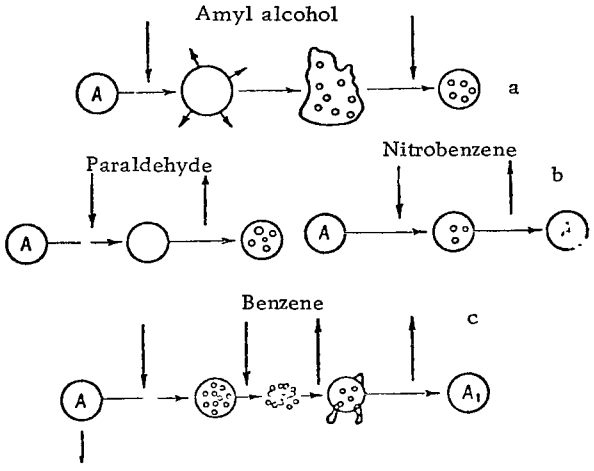


Figure 16. Change in the Shape of Coacervate Drops (a-c) Under the Influence of Nonelectrolyte Vapors.
A-A_1 — Initial and Final Drops; Downward Arrow - Nonelectrolyte is Added; Upward Arrow - Nonelectrolyte is Removed.

Figure 17, c, shows the changes undergone by a 3-layer coacervate drop obtained with the participation of aniline [514, 534, 560].

Surface layers are formed with particular case in lipoprotein coacervates, givein a so-called coacervate envelope.

Envelopes of Coacervate Drops. Many coacervate drops have thin surface layers of various compositions [201, 305, 306, 609, 654]. There is no single term designating these layers. They are called films, membranes, envelopes. From the standpoint of chemical composition they can be divided into lipid and nonlipid evelopes. /53

Salts of oleic acid - oleates and lecithin are used to prepare lipid envelopes. These compounds are most frequently classified among the so-called associated (associative) colloids whose micelles consist of different numbers of molecules [159, 366, 455, 522, 609].

TABLE 9. Effect of Alcohols on Coacervates

Alcohol	Amount of alcohol, mole/1, 3	Time of formation of deformed shapes, min
Methyl	1.04	27,0
Ethyl	0.96	12.7
Propyl	0.82	1.5
Butyl	0,26	1.25
Amyl	0.63	1.5

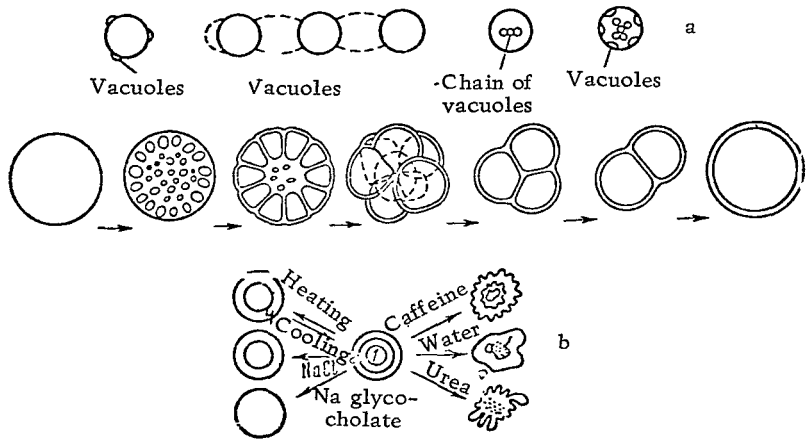


Figure 17. Formation of Vacuoles in Coacervate Drops.
a—Effect of Direct Current; b—Effect of Distilled Water; c—Change in the Shape of Coacervate Drops Under the Influence of Various Agents; I—Initial Three-Layer Drop Formed Under the Influence of Aniline.

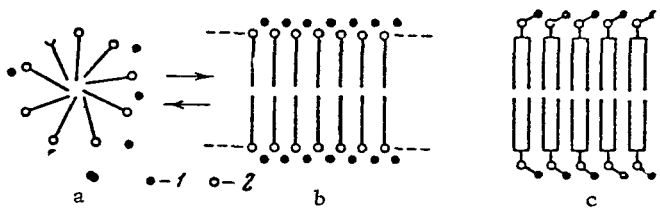


Figure 18. Transformation of Globular Micelles of Lipids into Linear Ones.
a—Globular Micelle of Oleate; b—Linear Micelle of Oleate; c—Linear Micelle of Lecithin; 1—Positive Charge; 2—Negative Charge.

Of great importance in this case is the ability of spherical micelles to convert into linear ones and to orient themselves in a definite manner on the surface of the drop, forming films of simple and complex structure.

Figure 18 shows the transformation of a spherical micelle of oleate and phosphatide into a linear one under

the influence of salts. This phenomenon, which is observed at a specific salt concentration, is reversible. The hydrophilic ends of the oleate molecule ($R-C\begin{matrix}O\\O\end{matrix}$) are oriented toward the aqueous medium outside, while the hydrocarbon radicals are oriented toward the inside of the micelle. When films are formed on the surface of the coacervates, the orientation of the molecules changes, and they produce a double layer. If phosphatides are present instead of the oleate, they also produce similar layers. Figure 19 shows the chemical formula of lecithin and the corresponding conventional representation of its molecule. Lecithin can participate in the formation of various types of films. The films can be obtained from mono-, di- and polymolecular layers of lecithin, from lecithin with the participation of calcium ions and protein substances. The chemical composition and the degree of concentration and contraction of the film are of major significance for the permeability, stability and many other properties. This can be demonstrated by using the following example. Let us consider a poision which contains the enzyme lecithase A (A-acylhydrolase of phosphatides 3.1.1.4) [176], which hydrolyzes lecithin with the formation of lysolecithin. Lysolecithin has a hemolytic effect, dissolving erythrocites. If the poison is added to an artificial lecithin film having 1.04 molecules per 10^{-14} /54
cm^2, 50% of the lecithin decomposes in 30 sec. Upon concentration, i.e., contraction to the film to 2.11 molecules of lecithin per 10^{-14} cm^2, the half-decomposition time increases to 3 min. Addition to protein to the lecithin film has an even more pronounced effect. In this case, a lecithin-protein film is formed which substantially inhibits the action of the poison [320]. Usually, the protein molecules in such films have a linear configuration. Figure 20,a, shows a coacervate envelope or film, and Fig. 20,b, a modern diagram of the structure of protoplasmic membranes according to Daniel [18, 106, 229, 361, 379, 388, 398, 649].

In a "sandwich" type biological membrane, lipids are sandwiched in-between two protein layers. The membrane is so similar to the Bungenberg de Jong model that it does not require any further clarification.

The existence of such membrances is possible owing to the presence therein of oppositely charged chemical groups [234, 460].

If vacuoles are present in a coacervate drop, they are separated from each other by films which, depending on the chemical composition and structure, /55
have different permeabilities. If the film is charged negatively and calcium ions are present in the liquid around the film, a new coacervate with adsortion of calcuim is formed. Calcium reinforces the attraction between the particles of the film, and the penetrability of the water decreases. The addition of sodium chloride enhances the penetrability of water through the film. In this case, the sodium and calcium ions behave as antagonists. By becoming located between the carbon chains of lecithin, cholesterol thickens the envelope, attracting the hydrocarbon groups to itself. At the same time, cholesterol acts as a kind of

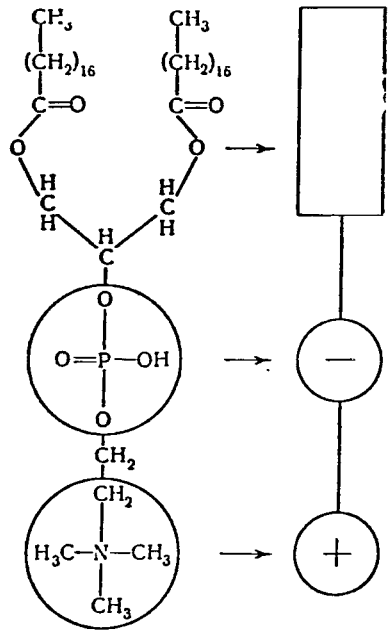


Figure 19. Chemical Formula and Concentional Representation of Lecitin.

pore, and those substances which are soluble therein can freely penetrate through the film. Like cholesterol, other compounds can also be included in the film. The films are most stable at pH values close to the isoelectric point of proteins and lipoids. A membrane type envelope is charged and characterized by a definite oxidation reduction potential. Systems of possible membranes have been described in detail by Bungenberg de Jong [539, 510, 600—602].

Obviously, not only vacuoles but liquid structure present inside the protoplasm are separated from the plasma by a layer in which the chemical substances are oriented in a definite manner. Cases may also arise where another coacervate which acts as an envelope becomes located on the surface of mixed coacervates. For example, if the coacervate from lecithin and $CaCl_2$ is added to the coacervate from gelatin and nucleic acid, the lecithin coacervate coats the coacervate from gelatin, forming a film envelope [475].

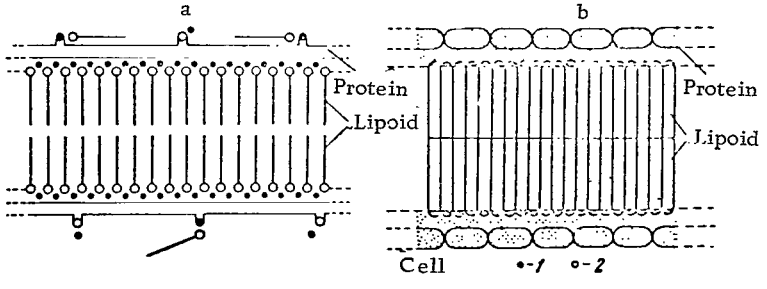
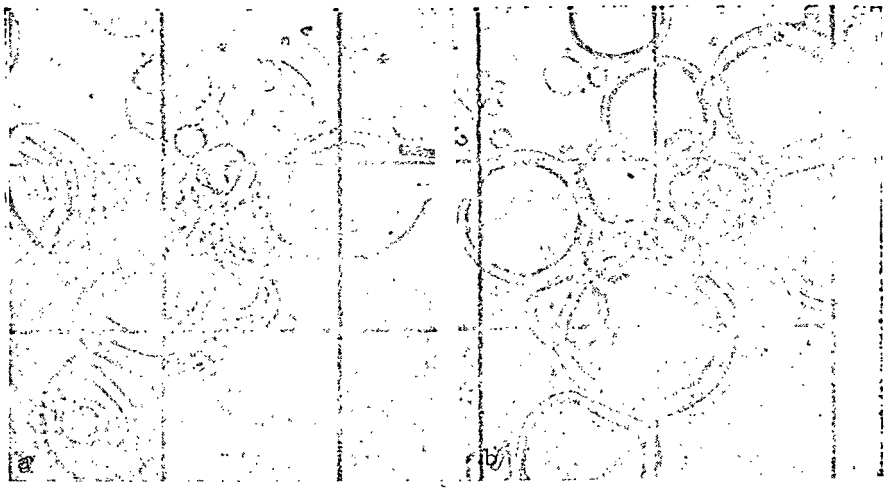


Figure 20. Diagram of Membranes.

a—Membrane of Coacervate Drop (after Booij and Bungenberg de Jong); b—Cell Membrane (after Daniel); 1—Positively Charged Groups; 2—Negatively Charged Groups.

Thus, a certain analogy can be drawn between coacervate films and cell membranes.

The problem of composition of envelopes is a great importance for preserving compounds in spheres and for their interaction with molecules of the ambient medium.



/56

Figure 21. Protein-Carbohydrate Coacervate Drops (a and b) with Envelopes (after Bungenberg de Jong).

Goldacre writes that the concentration of substances in the form of lipoprotein films could have taken place on the water surface of the primeval ocean, where he observed lipoprotein films. Under the impact of the waves, such films produced microspheres, i.e., bubbles which the author ascribed to coacervates [692, 834, 836].

Coacervate drops with nonlipid envelopes can be obtained from proteins and nucleic acids in combination with other compounds.

The structure and properties of such films have been studied very little. Obviously, they may differ from lipoprotein membranes. As an example, Fig. 21 shows protein-carbohydrate drops as envelopes were formed under the influence of a 30% sucrose solution. Drops consisting of gelatin and tannin have fairly dense envelopes. A method for preparing such globules was described by Bungenberg de Jong [504, 510, 589, 591, 613].

The formation of spheres in coacervates containing nucleic acids was demonstrated by Bungenberg de Jong [569,579].

V. G. Kryukov obtained "capsules" formed by the action of nucleic acids on proteins (edestin, egg albumin, globin, etc.). Kryukov holds that these capsules are of a coacervate nature. The same kind of capsules with vacuoles inside were

successfully observed in the interaction of nucleic acids with alkaloids: strychnine, curarine, caffeine and also certain dyes [193—195].

In many protein-nucleic coacervate drops, the boundary layers cannot be observed under ordinary microscopes. The study of coacervate drops by means of electron microscopes is only beginning. /57

The electron microscopic study of coarse coacervate drops, which has been the object of particular attention, is very difficult. Drops over 10 μ in diameter contain up to 50-90% water and may cause the appearance of new structures (artifacts) owing to the conditions of recording and photography in the electron microscope [14, 207, 406].

Recently, studies by I. G. Stoyanova et al. [367] have appeared which showed the feasibility of using electron microscopes with a humid chamber for live specimens without drying.

Some preliminary data on the ultrastructure of such drops, studied under the electron microscope, were obtained by Oparin, Stoyanova and Serebrovskaya [273, 329]. It was found that coacervate drops of serum albumin - gum arabic - RNA and ribonuclease are homogeneous under the standard microscope, whereas under the electron microscope they exhibit a definite structurized character, graininess and channels.

Figure 58 (cf. p. 144) shows a series of photographs of such drops obtained with an electron microscope.

The magnification on these pictures is obviously insufficient to provide an answer to the question of the boundary layer.

We therefore took finer drops from histone-RNA and clupein-RNA coacervates no more than 1.3 mμ in diameter. The drops contained 63-74% of dry matter and 26-37% of water.

Figure 22 shows photographs of drops obtained by Shurygina and Belyayev at the electron microscopy laboratory of Moscow University. Analysis of these photographs shows that the molecules are collected in separate clusters and that a boundary surface layer may exist.

The arrangement of the molecules in these structures is as yet unknown.

More detailed studies of the fine structure of the drops make it possible to compare their structure with that of protoplasm, observed under the electron microscope.

Fox, Harada, Fukushima, and Young, who have dealt with problems of the origin of life [288, 390, 391, 698, 962], obtained interesting microspheres with a strong proteinoid envelope. The method of preparation of the microspheres consists of the following.

A mixture of different amino acids with a definite content of asparagic and glutamic is subjected to thermal treatment of 120° in the absence of water. Polymerization forms proteinoids. In the presence of polyphosphoric esters, the process may take place at lower temperatures [680, 711, 713, 890]. The /58
protenoids are then treated with a 1% aqueous solution of NaCl with heating or boiling in order to obtain a saturated solution.

The microspheres are precipitated from the solutions on cooling. Attempts to obtain such microspheres from ordinary proteins have not yet been successful. This is because proteinoids differ from natural proteins in many properties, for example, the lack of optical activity, a low solubility, the presence of cyclic structures, etc.

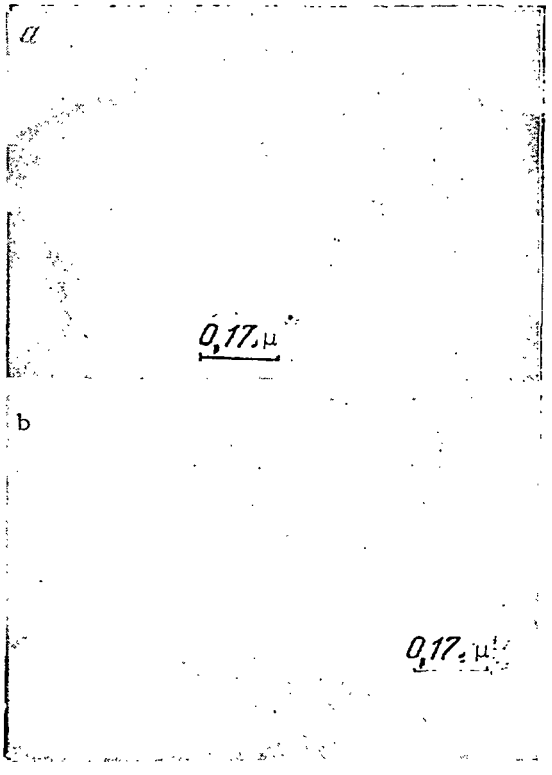


Figure 22. Appearance of Coacervate Drops Under the Electron Microscope.
a—Part of Drop of Clupein-DNA;
b—Part of Another Drop of Histone - RNA.

Microspheres are dense globules more or less uniform in size and characteristic of each proteinoid.

Fox suggests that the ability of /59
protenoids to produce microspheres is due to the properties of the individual amino acids. It is well known that under certain conditions of recrystallization, certain amino acids, for instance tyrosine, precipitate in the form of globules. A melanin polymer obtained from a mixture of tyrosine with phenylalanine also gives spheroids 1-2 mμ in diameter [469, 681]. Microspheres from proteinoids have the following properties: 1) birefringence in a flow, 2) the ability to swell and contract under the influence of salts, 3) septums are formed in them under pressure, 4) depending on the composition, they produce gram-positive or gram-negative forms, 5) the contents of the microspheres can be extracted by means of buffer solutions, and other compounds such as lecithin can be introduced into the hollow

microspheres; 6) microspheres containing ATP are capable of accelerating the cleavage of ATP with the formation of inorganic phosphorus; 7) they can absorb various compounds from the surrounding medium; 8) they show a good resistance to treatment with fixing agents, which are used to prepare sections. Such sections are then studied under the electron microscope [391, 681, 711—713].

Figure 23 shows the form of the microspheres under an ordinary microscope and sections of microspheres studied under an electron microscope at a low magnification. The microspheres have a distinct two-layer envelope and grainy contents.

Fox holds that proteinoids could have been synthesized on the earth as a result of the thermal energy of volcanoes, which together with other forms of energy could have been used for the abiogenic synthesis of chemical compounds [417, 418, 489, 663, 677—681, 750, 779, 820, 838, 922]. Such proteinoids then produced spheres which fell into the ocean and became more complex. Microspheres of such type are encountered in meteorites. Furthermore, they have been obtained by the action of electric discharges on a mixture of H_2O, CH_4, NH_3 and other compounds [698]. Fox is using microspheres as stable prebiological models.

Stability of Coacervate Drops. Since coacervate drops are liquid colloidal formations, the question naturally arises to what extent these changes occuring in drops and the drops themselves are stable and whether they can be preserved for a long time.

If coacervates are obtained in large quantities in vessels of low surface area and considerable height, after a period of time the coacervate drops deposit on the bottom, fuse and form a layer [280, 376, 461].

The time during which the drops change into layers depends on the composition of the coacervate and the size of the droplets.

If a small amount of coacervate is taken and placed in a thin layer by putting it in a close glass cell 0.15-0.20 mm thick and prevented from drying, the fusion /61 of the drops does not occur. The drops are preserved for several weeks and longer.

A very interesting study of the preparation of stable coacervate drops was made by Makovskiy, Steopos and Chaushesku [223].

The authors undertook a study of the structure of coacervate drops by methods employed in cytology.

They obtained coacervate drops from blood serum and gum arabic at pH 5.0. The drops were large, and many had vacuoles. They were found to be quite

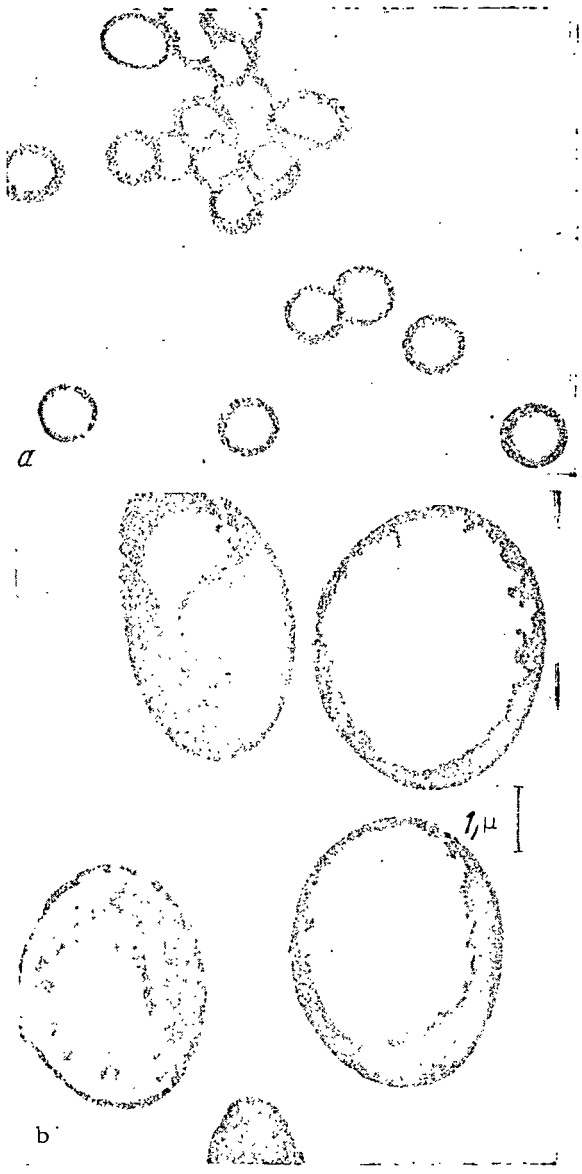


Figure 23. Microspheres of Synthetic Proteinoids (after Fox).
a—Appearance Under Ordinary Microscope; b—Appearance Under Electron Micorscope.

complex in chemical composition, and in addition, droplets of sunflower oil were easily introduced into them.

Upon addition of fixing agents employed in cytology to the drops, the latter broke apart. If the drops are initially placed in the sucrose solution, they become stable and can be treated with fixing agents. after the fixing, the drops behave as elastic globules. As we know, sucrose is widely employed for preserving ribosomes, mitochondria, nuclei, nucleoli and other structures when they are separated from cells [395, 732].

In concentrated sucrose solutions, the globules contract and assume different forms with many inclusions. In distilled water, they swell and assume the round shape of cells with a single central vacuole. The behavior of the droplets resembles the characteristic changes of protoplasm under the same conditions [222, 223].

The acquisition of stability by the drops, considered in its evolutionary aspect, was of major importance for their further improvement on the path toward the formation of forms directly preceding life [249—250].

Method of Preparation of Stable Coacervate Drops. To 1 ml of blood serum are added 1 ml of a 1% gum arabic solution, 0.6 ml of 20% acetic acid, and 7.4 ml of a 50% sucrose solution. A turbid solution with many coacervate drops of different sizes is formed.

In order to fix the coacervate obtained, to 10 ml of the suspension is added 1 ml of the fixing agent, consisting of a formaldehyde solution (40%), a 2% solution of osmium oxide, etc. The fixation lasts 24-28 hours. During this time, the coacervate drops deposit on the bottom of the vessel and do not fuse into a layer. The deposit is separated from the liquid by centrifuging. To the deposit are added 10 ml of a 50% sucrose solution, and the mixture is centrifuged for 5 min at 1000 rpm. Washing with a 50% sucrose solution is repeated 3 to 4 times. To the deposit containing fixed coacervate drops is added a warm 2% solution of agar, and the mixture is quickly cooled to 3—4°. After solidifying, the material is removed from the test tube and cut into 3-5 mm cubes. The cubes are dehydrated, as is done in cytology, by treatment for 1 hour in ethyl alcohol of increasing strength (30—80—96—99°). The tubes are then placed in benzene for 2 hours. This procedure is repeated 2 or 3 times. They are then soaked in paraffin for 3 to 4 hours at 59°. After cooling, sections of different thickness /62
are prepared from the cubes and stuck on glass with a protein-glycerin mixture prepared according to Mayer. The sections are dried, the paraffin is driven off with xylene, and the preparation is immersed in Canada balsam or cedar oil. The coacervate drops have a diameter of 30 μ and higher, and for this reason one drop of coacervate can be readily cut up into 3 to 8 parts.

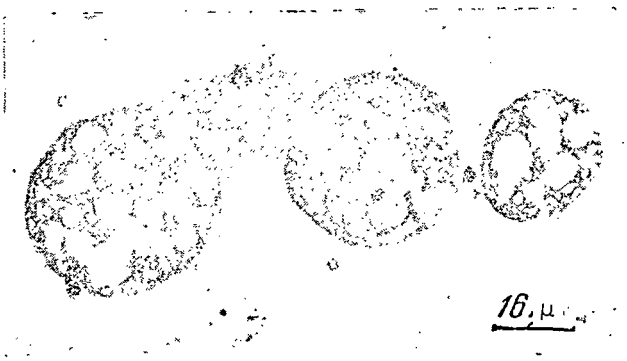


Figure 24. Cross Section of Coacervate Drops of Blood Serum (after Makovskiy).

Sections of stable drops can be studied under the microscope at various magnifications (Fig. 24).

A microscopic study showed that the drops have a well-formed envelope, vacuoles, and various inclusions.

At this time, it is difficult to say which appeared first in the process of evolution-spheres with strong envelope filled with coacervate contents, or drops which acquired a dence envelope by reacting with various compounds. It is possible that both of these processes took place at the same time.

It is generally known that the separation of polymers from solutions frequently occurs in the form of precipitates, gels and crystals of a given form with a distinct orientation of the molecules, for example, crystals of proteins, etc. [166, 169, 324, 807, 933].

A special form of the orientation of molecules is also characteristic of colloidal formations arising from inorganic and organic substances, possessing

anisotropy, and referred to as tactoids. Tactoids have a spindle shape with different degrees of elongation [319, 371, 409]. Bernal and Fankuchen [33,34, 90, 100, 208, 468, 737, 763] showed that the protein of tobacco mosaic gives tactoids. As the size of the tactoids increases, they approach a spherical shape.

One of the essential characteristics of coacervation is the capacity to form drops of various shapes and structures which is not limited by the preservability of the crystals.

In some of their properties, coacervates are similar to protoplasm. Therefore, when compared to other models, they have a number of advantages from the standpoint of the understanding of biological processes and of the structure of protoplasm. /63

No coacervate with a composition and structure as complex as those of a cell has as yet been prepared.

Protoplasm is a heterogeneous liquid system in which various types of liquid structures are present. These structures can be converted into each other under certain conditions.

Molecular Structure of Coacervates. Without an ordered arrangement of the molecules in the individual structures and boundaries between them, it is difficult to visualize the coordination of the action of enzymes in a cell and hence life itself.

The formation of the simplest structures and their evolution have been thoroughly analyzed by Bernal and other authors [33, 320].

There are three basic types of state of matter depending upon the arrangement of their particles (molecules, ions, atoms): 1) amorphous-isotropic, 2) paracrystalline, 3) crystalline.

Transition stages between these categories exist.

In some parts of the protoplasm of various cells, the substances may be present in all three states. The paracrystalline structure is the most characteristic of protoplasm. Thanks to electron microscopic studies and x-ray investigations it has been possible in many cases to determine the order of arrangement of individual groups of molecules in protoplasmic structures.

The molecular organization of protein, carbohydrate and nucleic coacervates has been studied insufficiently. There exist only a small number of studies devoted to the investigation of the configuration and arrangement of molecules relative to each other in such systems. It is known that in dilute aqueous solutions, molecules of proteins and nucleic acids most frequently have an elongated form.

It has been shown that the coacervation is associated with a transformation of unfolded (α-spiral) protein molecules into spherical ones [455]. Zimm observed a considerable decrease in the number of elongated α-spirals and their conversion into tangled balls as the concentration of the molecules was increased in gelatin solutions [400]. Interesting observations have been made recently by Izmaylova et al. [160, 632] who studied gelatin jellies with high protein concentrations. It was found that the conversion of gelatin molecules from α-spirals into balls takes place at a definite temperature. In coacervate drops and layers, the substances are concentrated. Therefore, the majority of the molecules may be assumed to be present in the form of balls [114, 759]. A relationship between the amount of α-spirals and balls can change, since the concentration of the substances in the drops varies considerably with their size and chemical composition.

Structured drops may contain vacuoles filled mainly with water. There are few protein molecules in vacuoles, and in this respect they are similar to the equilibrium liquid surrounding the drops. /64

Veis has shown that in the equilibrium liquid, the molecular weight of the gelatin is 3.3×10^5, and in the coacervate layer, $1\text{-}2 \times 10^6$. Thus, simultaneously with the increase in the concentration of molecules, an increase of their molecular weight took place [940].

The following data are available on the order of arrangement of molecules in coacervate layers and drops.

By measuring the elastic-viscous properties of a protein coacervate layer and jellies, Pchelin and Solomchenko observed an ordered arrangement of molecules in the coacervate layer. However, the structure of the layer differed from the structure of the gels [307].

Another proof of the presence of a structural state of the molecules is the determination of the birefringence in liquids.

Solutions of low concentrations of the substances are used to prepare coacervates [776]. The molecules move in a random fashion in these solutions, this being characteristic of the liquid isotropic amorphous state of matter.* The capacity for birefringence is manifested in systems where the molecules are oriented in a definite fashion. It is lacking in the presence of random motion.

*Kargin et al, [167] made a close study of the structure of amorphous polymer precipitates under the electron microscope and showed that such precipitates contain not only a disordered arrangement of molecules but also a structural and ordered arrangement.

Coacervate layers show birefringence in a flow. In this case, the molecules of the solvent, i.e., water, by pushing apart the particles of, for example, protein substances, orient them in a given direction. In coacervate layers, the number of molecules is large, and hence, the distance between them decreases considerably.

For this reason, the forces of attraction between molecules increase, and the preservation of the orientation is facilitated.

The content of the substances is highest in the dense envelopes of coacervate layers obtained from tennin and gelatin, which also exhibit birefringence [510].

Thus, similtaneously with an increase in the concentration of molecules in the layers or drops, structures characteristic of the paracrystalline state of matter arise in coacervates.

The relationship between the concentration of molecules and their capacity for structuration has been amply demonstrated by Luzzati and Nicolaieff. Using x-rays, they analyzed the structure of model spherical particles obtained by mixing solutions of histone and protamine with DNA. The mechanisms which they give demonstrate a gradual transition from single fine particles to spheres having a definite structure. At a low concentration of the substances, individual nucleoprotein particles are formed. When the particles consist of four DNA molecules surrounded by histone, they are arranged at random. An increase of the concentration of the substances first causes an increase of their number, and then fusion into ordered hexagonal structures. The authors emphasize that in addition to the chemical nature of the substances, of great importance for the decomposition of the molecules in such spheres and for the size of the spheres themselves in the concentration of the starting compounds and mineral salts [218,781,964]. /65

The diameter of large spheres is 10^{-6} cm, and that of coacervate drops of these substances, 10^{-5} cm and higher.

The problem of the relationship of the structure and concentrations of the compounds from which they are formed is of prime significance. The concentration of substances is a basic property of coacervate systems. The quantitative aspect of this problem as well as the distribution of the compounds in homogeneous and structured individual drops are examined in the following chapters.

Chapter 4

CONCENTRATION OF SUBSTANCES IN COACERVATE DROPS

/66

Coacervation is associated with the concentration of substances in drops. In a coacervate system, the volume of all the drops is tens of times less than that of the surrounding equilibrium liquid. Nevertheless, over 70% of the dry matter of the substances is contained in the drops. If the content per 1 ml of drops is compared with the content per 1 ml of equilibrium liquid, over 90% of the dry matter is concentrated in the drops, and only a minor portion remains in the equilibrium liquid. This residue consists mainly of low molecular compounds which most frequently are uniformly distributed between the coacervate layer, the drops, and the equilibrium liquid [129, 131, 325, 413].

Thus, coacervate is characterized by an increase in the concentration of the dry matter in the drops by a factor of several tens of times, as compared to the original solutions, and even more as compared to the equilibrium liquid, reaching a 100-fold value. This difference is due to the fact that the concentration of the substances in the equilibrium liquid is always lower than in the initial solutions, since the chemical compounds collect in the drops.

Such results have been obtained in studies of protein-carbohydrate, protein-protein, protein-nucleic coacervates and coacervates even more complex in chemical composition. For instance, in the coacervate from histone-DNA, 100 mg of dry matter was contained in the drops, and 3.7 mg in the equilibrium liquid. Consequently, 96.3% of all the compounds were contained in the drops. The concentration of the substances in the drops increased to 10%, while it was about 0.1% in the initial solutions from which the drops were obtained. These results are typical of all drops or coacervate layers [529]. Even if this substance separated in the form layers as a result of standing or centrifuging [211, 250, 376, 508, 521, 557].

/67

At the same time, individual drops differ from each other in size and structure. Obviously, the content and concentration of substances in different drops are different. Chemical methods cannot be used to weigh a droplet. Most suitable for these purposes is a physical method, quantitative interference microscopy or interferometry, which involves the use of light beams permitting the weighing of the substances of drops and cells down to 10^{-13}– 10^{-14} g.

This method has now provided data on the concentration of substances and on the dry weight of cells and subcellular structures: nuclei, nucleoli, etc. [19, 444—446].

Characteristics of Interference Microscopy as a Method for Determining the Dry Matter of Substances Down to 10^{-14} g in Drops and Live Cells

The founders of interference microscopy were Lebedev and Linnik. In 1920-1924, Lebedev built the first polarization interferometer arranged like a microscope, and in 1933-1934 Linnik created a model of an interference microscope for opaque objects. Basic data on interference microscopy can be found in the papers of Zakhar'yevskiy and Kuznetseva et al.[10, 155—157, 381—382, 667, 682, 721, 806, 817].

The essence of the method of interference microscopy consists in the following. Any microscopic object whose refractive index differs from that of the surrounding medium causes a shift in the oscillations of the light wave going through it both in phase and in amplitude or in one of these. The amplitude shift takes place when light beams are absorbed. This usually takes place in the case of colored objects. For colorless ones which are transparent in visible light, the change in amplitude is so slight that it is neglected when the phase shift is measured in interference microscopes. Form the phase shift, the content and concentration of the substances in microscopic objects is calculated. The phase difference, measured in μ or cm, also called the path difference, arises in interference microscopes as a result of the fact that one part of the interfering rays goes through the object and the other part through the medium. Figure 25, a-c show diagrams of the path and vibrations of rays through a coacervate drop and the medium surrounding it, and also shows a simple diagram of the path of the rays in interference microscopes.

An examination of Fig. 25, a, b, c, showed that the two rays S_1 and S_2 /68
travel from the light source J through the preparation containing the coacervate. Ray S_1 passes through the medium surrounding the drop and emerges as ray S_W. Ray S_2 penetrates the drop, is refracted in it, and emerges as ray S_0. As a result of the refraction, the path of ray S_0 will be longer than that of ray S_W, and the two will arrive at the same point at different times. Ray S_W will reach this point earlier, and ray S_0 will lag behind. If the vibrations of the light wave are represented as a sine wave and the time t is laid off along the obscissa axis, then for ray S_W the wave will begin at point A and end at point B, and for ray S_0, respectively at points A_1 and B_1. The segment $A—A_1=\delta$ and shows how much the vibration of ray S_0 lags behind ray S_W. In this case, this lag amounts to 1/4 of a wave, and this difference will be constant over /69
the entire course of rays S_W and S_0. Despite the fact that the coacervate was placed in a glass cell, the refraction of rays S_1, S_2, S_W, S_0 on going from air through the glass of the cell and emerging from the latter will be the same and will cause no phase change in one ray as compared to the other. Hence, the phase difference will be caused only the passage of one of the rays through the drop and the other through the medium. It should be emphasized that the more

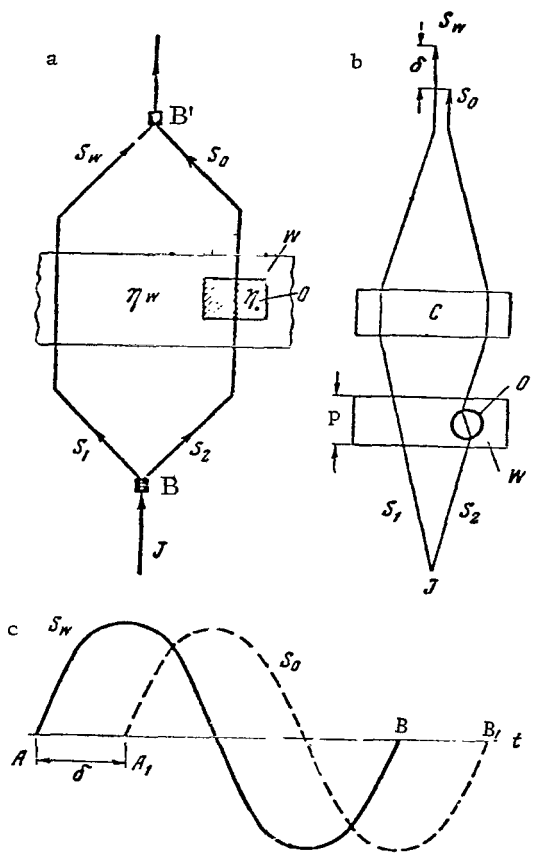


Figure 25. Path of Rays.

a—In the Interference Microscopes; b—Through Coacervates in Interference Microscopes; c—Phase Change; δ—Path Difference (of Phases); p—Preparation; C—Condenser, J—Source of Rays; S_1 S_2—Interferring Rays; W—Medium; S_W—Ray Which has Passed Through the Medium; AB-A_1B_1—Wavelength; t—Time; S_0—Ray from Light Source; B—Point of Splitting of the Rays; B'—Point Where the Rays are Reunited; O—Object; η_0 Refractive Index of Object. η_W Refractive Index of the Medium.

substance is contained in the drop, the stronger the refraction of the rays, so that an increase in phase difference results. The relationship between the refractive index of the ray and the phase difference is shown on a diagram of the path of the rays in interference microscopes (Fig. 25, a) and in formulas 1-3.

The light rays J shown in Fig. 25, a, are polarized, collected by a condenser and directed at the splitting point of interferometer A. On emerging from point A, one-half of rays S_1 pass through medium w with refractive index M_W, and the other, S_2, through object o with refractive index n_o. Rays S_W and S_0, which have passed respectively through the medium and the object, have different phases and are recombined by the condensing parts at point B; the phase difference is then measured with special devices. The relationship between the phases difference and the refractive index is expressed by formula (1) [162]

$$\delta = (n_0 - n_w) \cdot d, \qquad (1)$$

where δ is the path difference in cm; n_0 is the refractive index of the object; n_W is the refractive index of the medium; d is the thickness of the object in cm.

Knowing δ, one can readily calculate the concentration of the substance and its weight in the object using the following equation

$$n_0 = n_w + \alpha C,$$

where C is the content of the substance in 100 ml of solution; α is the specific refraction coefficient. In our case,

$$\delta = [(n_w + \alpha C) - n_w]\, d = \alpha C d \tag{3}$$

$$C = \frac{\delta}{\alpha \cdot d} = \frac{P \cdot 100}{V}; \tag{4}$$

$$P = \frac{\delta \cdot V}{100 \cdot \alpha \cdot d} = \frac{\delta \cdot S}{100 \cdot \alpha}, \tag{5}$$

where S is the area in cm^2; P is the weight in g; V is the volume in cm^3, and V/d = S.

Formula (4) will apply to the measurement of an object having a uniform thickness. For objects which do not have these properties, corresponding corrections are introduced. Formula (5) is very convenient for calculating /70
the weight, since the volume and thickness of the object do not have to be known when it is employed. In many cases, particularly of cells of irregular shape, the volume cannot always be calculated. Therefore, calculations of the dry weight per unit area of the object are frequently used for obtaining comparative data within the confines of cells of the same tissue or the same microorganisms.

In calculating the concentration as well as the weight, it is necessary to know the specific refraction coefficient (α), which depends on the chemical nature of the substance and can be found from formula (6) [445].

$$\alpha = \frac{n - n_S}{C}, \tag{6}$$

where n is the refractive index of the solution, n_S is the refractive index of the solvent, and C is the concentration of the substance equal to 1 g/100 ml of solution.

The coefficients of refraction α can be measured in both refractometers and interference microscopes with special attachments [163, 174, 443, 448, 449, 451, 647, 729].

The coefficient of refraction can change somewhat depending on the concentration of the solutions, pH, temperature and wavelength at which the reading of α is made.

The influence of these factors on α of serum albumin is shown in Table 10, and values of α for different substances are given in Table 11.

TABLE 10. Effect of Various Factors on the Specific Coefficient of Refraction of Serum Albumin

Factor	Refraction coefficient (α)	Source	Factor	Refraction coefficient (α)	Source
Wavelenght, mμ			Temperature, °C		
336	0.00198	[452]	5	0.001887	[856]
579	0.00183	[452]	20	0.001862	[856]
Concentration, %			pH		
2.9—8.5	0.001830	[442]	5.35	0.001857	[426]
5—55	0.001844	[452]	7.4	0,00187	[426]

The above data indicate that for proteins and nucleic acids, the value of α is equal to an average of $0.0018 \pm 2\%$. This value is used in calculating the concentration and weight of substances of cells and subcellular structures, since the bulk of the dry matter of the protoplasm is constituted by proteins [247]. At /74
the same time, mineral salts are indispensable components of cells. On the average. the value of α for mineral salts is 0.0016. The presence of salts has only a very slight effect on the coefficient of refraction of proteins and other compounds (only 0.00001), and is therefore neglected [444]. In the best models of interference microscopes and suitable objects, one can measure down to 10^{-14} g of substances and a concentration down to 0.02%. In most cases, the error in the value of the dry weight being determined amounts to 5-10%.

An essential step in working with cells surrounded by water is their physiological state. Different compounds can pass into water from dying and dead cells. The weight of such cells, measured in interference microscopes, will be lower than that of normal ones. The widely employed method of luminescence microscopy can serve as a method for checking viability [103, 228]. The luminescence of live cells in a luminescence microscope differs from the luminescence of injured cells [137].

TABLE 11. Specific Refraction Coefficients

/71

No.	Compound	α	Source
1	Human serum albumin (crystalline)...	0.00181	[426]
2	Human serum albumin	0.00186	[435]
3	Ox serum albumin.....	0.00187	[856]
4	Horse serum albumin.....	0.00184	[708]
5	Horse serum albumin.....	0.00183	[426]
6	Egg albumin.....	0.00182-0.001876	435, 856]
7	Human serum globulin.....	0.00181-0.00186	[426]
8	Horse serum globulin	0.00186	[426]
9	Human gamma globulin.....	0.00188	[426]
10	Euglobulin.....	0.00183	[426]
11	Pseudoglobulin.....	0.00181	[426]
12	Whole human blood.....	0.00179	[426]
13	Human plasma.....	0.00183	[435]
14	Lactoglobulin.....	0.001818-0.001890	[708, 853]
15	Ox globin	0.00178	[25]
16	Human hemoglobin	0.00194	[915]
17	Same as 16.....	0.00193	[426]
18	Ox carboxyhemoglobin	0.00193	[426[
19	Sheep carboxyhemoglobin	0.001945	[426]
20	Human fibrinogen	0.00183	[435]
21	Peptone	0.00183	[435]
22	Snail hemocyanin	0.00187	[869]
23	Mollusk hemocyanin	0.00197	[449, 452]
24	Crab hemocyanin	0.00187	[869]
25	Octopus hemocyanin	0.00184	[869]
26	Xiphosura hemocyanin	0.00198	[449, 452]
27	Lipoproteids	0.00170-0.00171	[426]
28	α lipoproteids	0.00178	[426]
29	β lipoproteids	0.00171	[426]
30	Nucleoproteids of tobacco mosaic virsus	0.00170	[647]
31	Nucleic acids	0.0016-0.0020	[442, 928]
32	Glycine	0.00179	[426]
33	Alanine	0.00171	[426]
34	Valine	0.00175	[426]
35	Tryptophan	0.00252	[426]
36	Glucose	0.00143	[647]
37	Sucrose	0.00141	[647]
38	Starch	0.00133	[647]
39	NaCl	0.0016	[647]
40	$CaCl_2$	0.0021	[647]

TABLE 12. Content of Dry Matter of Substances in Cells of Organisms (in g and %)

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No.	Specimen	Dry weight	Source
	Bacteria		
1	Escherichia coil.....	23.5-28%	[444]
2	Proteus vulgaris	25-30%	[444]
3	Bacillus subtilis	25-30%	[444]
4	Streptococcus faecalis ..	36-39%	[444]
5	Bacterial spores	55%	[814]
	Fungi		
6	Actinomyces bons	26-40%	[illegible]
7	Schizosaccharomyces pombe	23.6-26.3%	[illegible 2]
8	Saccharomyces cerevisiae	27-30%	[813]
9	Hyphae of zygomycetes ...	13-17%	[444]
10	Phycomycetes	28%	[444]
11	Penicillum notatum		
	Plasma	28%	[444]
	Spores	55%	
	Plants		
12	Bluish-green thermophilic seaweed, slimy sheath Mastigocladus laminosus Cohn.		[129]
	Mucilaginous sheath	5%	[129]
	Vegetative cells, size 26.1×10^{-8} cm^2	25%	[129]
	Reproductive cells, size 25.6×10^{-8} cm^2	29.6-30%	[129]
13	Tradescantia pollen ,.....	$0.5 \cdot 10^{-9}$ g	[648]
14	Rice pollen	$5.07 \cdot 10^{-9}$ g	[648]
15	Lawn grass		
	Spermatocytes	$41-66 \cdot 10^{-12}$ g	[956]
	Neuclei of spermatids	$19 \cdot 10^{-12}$ g	[956]
16	Melanopsis olifferentiallis ..		
	Diploid chromocytes	$31 \cdot 10^{-12}$ g	[956]
	Teraploid chromocytes	$65 \cdot 10^{-12}$ g	[956]
	Octoploid chromocytes	$116 \cdot 10^{-12}$ g	[956]
	Animal Cells		
	Animal cells have an average dry mass of	10-25%	[955]
		$2-400 \cdot 10^{-12}$ g	[955]
	Nuclei.....	$0.5-100 \cdot 10^{-12}$ g	[955]

TABLE 12. Content of Dry Matter of Substances in Cells of Organisms (in g and %) (continued) /73

No.	Specimen	Dry Weight	Source
17	Euglena perancma	28%	[444]
18	Amoeba verucosa pseudopodia.	10%	[444]
19	Amoeba radiosa psuedopodia ...	15%	[444]
20	Stylonichia of ectoplasm	23.5%	[444]
21	Mouse lymphocytes	14%	[865]
22	Snail amebocytes	13.8%	[447]
23	Sheep erythrocytes	$29 \cdot 10^{-12}$ g	[715]
24	Human erythrocytes	$31.4\text{-}32 \cdot 10^{-12}$ g	[442]
25	Sheep spermatozoa	$6.3\text{-}7.4 \cdot 10^{-12}$ g	[648]
26	Rat spermatozoa	$11.0 \cdot 10^{-12}$ g	[794]
27	Guinea pig spermatozoa	$11.5 \cdot 10^{-12}$ g	
28	Eye rods	40-43%	[896]
29	Human skin cells	$2.5\text{-}3.0 \cdot 10^{-12}$ g/10^{-8} cm^2	
	Outer layers of epithelium	30%	[451]
	Inner layers of epithelium	14%	[451]
30	Dog mucosum	$0.8\text{-}1.0 \cdot 10^{-12}$ g/10^{-8} cm^2	[646]
31	Human str. mucosum	$0.9 \cdot 10^{-12}$ g/10^{-8} cm^2	[646]
32	Tissues of rat aorta	$1.7\text{-}1.8 \cdot 10^{-12}$ g/10^{-8} cm^2	[646]
	Cell Cytoplasm		
33	Rhizopoda liebrekunia	4.7-5.5%	[444]
34	Gronia	5.5-6.3%	[444]
35	Salamander	8-12.5%	[444]
36	Viper	11.4-11.7%	[444]
37	Locusta nigratoria spermatocytes	10.7%	[872]
38	Liver cells	11.7%	[691]
39	Sea urchin	23%	[818]
	Cell Nuclei		
40	Locusta nigratoria spermatocytes	20.8-24.5%	[872]
41	Sea urchin, unfertilized egg	16%	[818]
42	Rat parotid gland	$2 \pm 2 \cdot 10^{-12}$ g	[55]
43	Extra-orbital gland	$42 \pm 3 \cdot 10^{-12}$ g	[55]
44	Nucleolus of motoneurons	70%	[55]
45	Nucleolus of liver cells	37% (± 2%)	[913, 914]
46	Mitochondria	$0.6801.78 \cdot 10^{-13}$ g	[765]
	Tumor Cells		
47	Hepatoma cells	16.3%	[691]
48	Ascites cancer	$29.4 \cdot 10^{-12}$ g	[921]

As an example, Table 12 lists some figures on the concentration and content of the dry matter of cells and subcellular structures.

It follows from the data of Table 12 that spores have the highest density. The lowest dry matter value was obtained for vacuoles and Golgi structure, consisting of a system of membranes and bubbles [444—450, 751, 818].

It is interesting to note that nuclei can apparently contain even fewer substances than cytoplasm [818]. Ross [872] gives the following results of measurements of the dry matter and nuclei of spermatocytes of Locusta nigratoria during their growth.

Volume of nucleus, 10^{-12} cm^3	Concentration of dry substance, %
4.2	24.5
4.7	23
5.3	21.9

For the case of chicken fibroblasts, Davies showed that as the area of the nucleus increases, the weight per unit area decreases [647].

Alfert et al. studied the influence of various factors on the size and dry weight of nuclei of follicular epithelium of rat thyroid gland. Small nuclei were obtained under the influence of hypophysis hormones, and large ones by adding propylthiouracil. Cells with normal nuclei were used as controls [428—429, 438]. The results of these experiments were shown in Table 13.

Hence, as the size of the nuclei increases, they become diluted, although the total content of the dry substance increases. The same is observed for nuclei of sheep sperm heads. In large heads, the concentration of the dry matter was found to be greater than in small ones [647].

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TABLE 13: Changes in nuclei of follicular epithelium of rat thyroid gland

Size of Nucleus	Volume, 10^{-12} cm^3	Histone Content, 10^{-12} g	Histone Concentration, %
Small	39	266±13	6.8
Normal	78	272±14	3.5
Large	110	256±11	2.3

Roels showed that under the influence of various factors, a change in volume and weight takes place in the nuclei of the renal membrane. As the volume of the nuclei increased, their concentration of substances decreased [871].

Relationships of this kind can also be observed under the influence of various agents altering the permeability of cells. For example, under the influence of

estradiol, which increases the permeability of epithelial cells of the bladder of mice, the volume of the cells increases as a result of the increased supply of water and decrease of the concentration of substances [829].

Erythrocytes act as osmometers: as the volume decreases, water is lost, and the density of the cells increases; conversely, by soaking up water, the cells increase in volume, and their concentration of substances decreases [375—377, 662, 766, 934].

Of major interest are data on the change in the content of dry matter in the course of growth, division and development of cells and unicellular microorganisms. It should be noted that for unicellular forms, the figures are more accurate than for cells located in tissues and surrounded by other cells because of the distortion of the interference pattern.

Studies on the cycles of development of unicellular organisms were made by Mitchison, Passano et al., and other investigators. Figure 26 shows changes in the volume, weight and concentration of substances in the course of development of bacteria and fungi [227, 813—818, 883].

In all cases, at the precise moment of cell division, the synthesis of substances and an increase of their concentration take place. The cell grows very slowly during the period. This is followed by a period of rapid growth and increase in the size of the cell. The concentration drops, reaching its lowest level prior to cell division.

The same was demonstrated by Grundmann [700] for the case of regenerating nuclei of liver cells (Fig. 26, d) and for HeLa cells growing in a tissue culture by Sandritter [882].

Bacteria. In Streptococcus faecalis prior to the division, the volume of cell is 5.56×10^{-12} cm^3, and the concentration of substances, 29%. After the division, the volume of the daughter cell is 3.44×10^{-12} cm^3, and the concentration is increased to 36%. Changes in cells of Tetrahynema pyriformis studied by Zeuthen [963] followed the same lines. /77

Fungi (Yeast). The cells of Schizosaccharomyces pombe have a diameter of 8.5×10^{-4} cm. Prior to division, they increase to 16.10^{-4} cm, and this is associated with a considerable increase in volume. At the same time, an increase of the total weight of the cell and a decrease of concentration are observed. Only the septum manages to form, since the concentration begins to increase again, i.e., the content of dry matter per unit volume of the cell increases; small cells are denser than large ones. The same kind of data were obtained for the growth of a culture at both 37° and 17° [818]. Hence, the increase in cell volume is due not only to the increase of the total content of the substances,

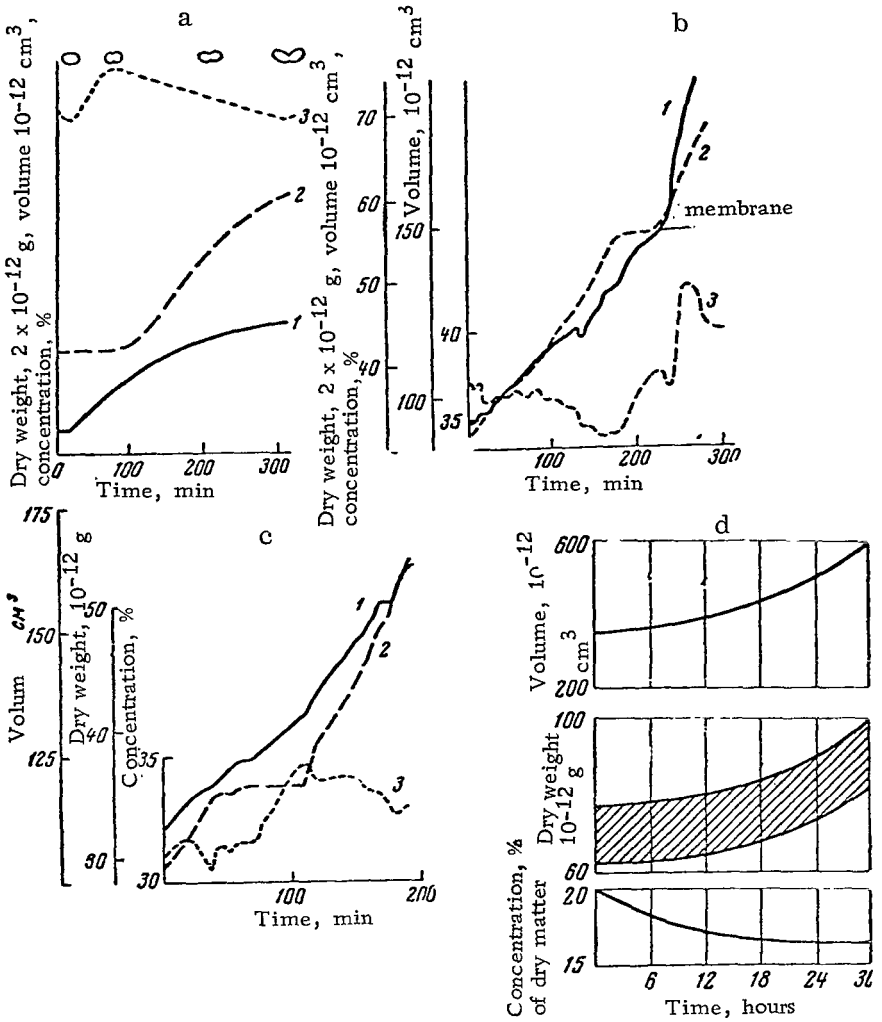

Figure 26. Change in the Concentration of Substances, Dry Weight and Volume of Cells During Growth and Division.

a—Bacteria of Streptococcus Faecalis; b—Fungi of Schizosaccharomyces pombe; c—Fungi of Saccharomyces Cerevisiae; d—Regeneration of Mouse Liver Cells; 1—Dry Weight; 2—Volume; 3—Concentration.

but also to the supply of water. Fluctuations in the concentration of substances range from 33 to 42% [814—816]. A similar picture was observed in Saccharomyces cerevisiae in both the first and second division of cells. Here the concentration changed from 30 to 35% [813, 850]. The maximum content of dry matter per unit volume was recorded 20 minutes after the division, and the minimum, after 40 minutes, before the next division of the cells, i.e., again when the cells had their largest volume.

Thus, yeast fungi and bacteria are characterized by an inverse proportion between the increase in volume and the content of dry matter per unit volume, but at the same time there is a direct proportion between the volume of the cell and its total weight. All these results were obtained on relatively simple specimens. A whole series of studies by Kimball, Caspersson, Svensson and others dealing with a quantitative cytochemical investigation of infusoria (Paramecium aurelia) by methods of interference and ultraviolet microscopy and also x-rays showed that the increase of weight in infusoria has a different course during its development, i.e., follows an exponential function [739—743].

Such differences are obviously explained by a more complex organization of infusoria as compared to bacteria. At different stages of ontogenesis, periods are observed when the size of individual cells remains unchanged, whereas the dry weight may increase or decrease as a result of synthesis, decomposition and discharge of substances into the surrounding medium [55].

Broad use of interference microscopy for the determination of the dry weight of cells began in the last decade. Therefore, in order to estimate the reliability of the data obtained, it is necessary to cite data on the weight of cells obtained by chemical, refractometric and x-ray methods [647, 961]. /78

Table 14 shows results of the determination of the weight of cells carried out by different methods.

TABLE 14. Content of Dry Mass in Cells (in g and %)

Specimen	Comparison of Methods		Source
	Interference	Refractometric	
Amebocytes of snail (Helix aspersa)	13.8%	13.5%	[447]
		Chemical	
Nuclei of cells of calf thymus gland	$19.8 \cdot 10^{-12}$ g	$19.1 \cdot 10^{-12}$ g	
	$21.1 \cdot 10^{-12}$ g	$23.0 \cdot 10^{-12}$ g	
	$35.0 \cdot 10^{-12}$ g	$33.0 \cdot 10^{-12}$ g	[705]
		X-ray	
Human skin cells	$2.5\text{–}8.0 \cdot 10^{-12}$ g/10^{-8} cm^2	$2.7\text{–}2.9 \cdot 10^{-12}$ g/10^{-8} cm^2	[645]
Human str. mucosum	0.9δ	10 δ	[646]
Dog mucosum	0.8–1.0 δ	0.8–1.3 δ	[646]
Rat Aorta	1.7–1.8 δ	1.3 δ	[646]
Bean tissues	1.98 δ	2.0–2.1 δ	[645]
Cells of ganglia of spinal cord (fixed according to the Carnois method)	$2.3 \cdot 10^{-4}$ g/cm^2	$2.4 \cdot 10^{-4}$ g/cm^2	[693]

Analysis of Table 14 shows that the discrepancies between the different methods are slight. However, some authors [846] obtained higher values for the dry matter content by photographing the specimens in x-rays as compared to the interference method [645—646, 826].

Gramp [693], who made a special study of this problem, showed that these high values, obtained when x-rays were used, where due to the emulsion layer of the photographic plates and to the methods used for fixing the material. Usually, large specimens are measured directly in interference microscopes, whereas fixed, dried or frozen specimens are used in x-ray methods. It is of course much more desirable to obtain data by direct measurements on a live specimen by fixation, since the latter frequently distorts the results.

Using an interference microscope, Mellors [804] studied the inference of fixation on the dry mass of rat spermatozoa. In live spermatozoa, 16 and 11.0 x 10^{-12} g of dry matter was found, whereas in fixed ones, respectively 13 and 9.8 x 10^{-12} g. Hence, part of the compounds were removed from the cells during the fixation. /79

The existing slight discrepancies between the chemical and interference methods in the determination of the dry mass of nuclei can be explained by the fact that different conditions of extraction of nuclei by chemical methods also affect their weight; this was examined in detail by Downs and Schwartz [889].

The latter found that the weight of nuclei extracted by nonaqueous solvents was greater than that obtained with aqueous solvents. Mitchison demonstrated the reliability of interferometry and ordinary weighing for the determination of the weight of Schizosaccharomyces pombe cells after the extraction of low molecular compounds from them [815].

The chemical method requires the investment of considerable time and material and gives only an idea of the average dry weight of the cell obtained from billions of cells [129].

Refractometry is more convenient and economical. However, in order to determine the refraction coefficients the cells are placed in various liquids which are not always inert toward live organisms.

Of great advantage over all the methods known at the present time is quantitative interference microscopy, which permits the determination of the weight of an individual unaltered live cell and its components in a short period of time.

In our view, because of its qualities, this method will find wide applications in the solution of not only scientific but also technical problems, for example the

determination of the full value and weight of plant tissues of agricultural crops, in medicine, and in the preparation of antibiotics, etc.

At the present time, interference microscopy has already found applications in many chapters of biology.

Use of Interference Microscopy. The qualitative and quantitative methods of interference microscopy have broad applications in histochemical studies.

1. To elucidate the localization of various compounds in cells [7, 65, 298], for example protein and RNA during division of sea urchin eggs [879], and also to elucidate the translocation of chromosomes and substances in various phases of division [805].

2. As a check of the growth and weight of cells in monolayers of tissue cultures [423, 882]. Thomson has described a special attachment to the interference /80
microscope permitting the carrying out of such studies [930].

3. In order to determine the configuration and thickness of cells [688, 704, 726] in this manner, the shape of human erythrocytes was determined. It was found that the erythrocyte is flattened at the center and has a thickness of $0.14-0.17 \times 10^{-4}$ cm, and $0.6-1.02 \times 10^{-4}$ cm at the edges [155, 648, 744].

4. For determining the dry matter of cells during regeneration and also in pathological changes resulting from traumas and diseases [700, 745] such as weight loss at various stages of hemolysis of erythrocytes [448, 644, 715, 794, 795, 803—805], decrease in weight and increase in the size of lymphocytes in leukemia (Table 15) [865].

TABLE 15. Change in the Dry Matter of Lymphocytes in Leukemia

Specimen	Healthy cells		Diseased cells	
	Diameter, 10^{-4} cm	Weight, 10^{-12} g	Diameter, 10^{-4} cm	Weight, 10^{-12} g
Mouse	7.1	13.5	8.3	11.4
Man	8.8	9.3	11.0	8.8

The relationship between the dry matter, volume and concentration of nuclei in regenerating liver cell tissue is shown in Fig. 26, d [700].

5. In studies of the influence of various hormones and dietary regime on cells, and also of cycles of their development [694, 691, 706, 778, 829, 870, 912—914].

6. For enzyme reactions, as a result of which a change in the content of substances is observed. For example, the determination of the activity of phosphatases in kidneys from the reaction of formation of Ca phosphate in cells. The action of phosphatases splits off the orthophosphate, which becomes bound to calcium salts added to the cell. Calcium phosphate deposits as a precipitate, and its accumulation is estimated from the increase in the wave path difference [454, 623, 642]. /81

TABLE 16. Change in the Dry Weight of Tradescantia Poll n Under the Influence of Enzymes (in 10^{-12} g)

Number of cells	Initial weight	Weight after the action of enzymes		Degree of hydrolysis, %	
		Ribonuclease	Trypsin	RNA	Protein
1st	4890	4210	1970	14	60
2nd	5070	4870	2270	4	55
3rd	5120	4500	1990	12	61
4th	5140	4910	2150	4.5	68

Table 16 shows the weight change of cells of nature pollen of tradescantia acted upon by ribonuclease, which cleaves RNA, and by trypsin, which hydrolyzes proteins.

Such studies are being made not only on plant but also on animal cells [913—914].

The main obstacles in calulating the weight of cells is the measurement of the volumes of cells or their areas [873]. The method of interference microscopy itself is simple.

Method of Determination of the Content of Substances in Individual Coacervate Drops*

The calculation of the concentration and content of substances in globular unstructured coacervate drops was made by using the formulas

*The study of the determination of the total content of substances in individual coacervate drops was made in the section headed by Professor A. N. Zakhar'yevskiy at the State Optical Institute im. S. I. Vavilov in Leningrad in cooperation with scientific collaborator A. F. Kuznetsova [129, 131, 140—142].

$$C = \frac{\delta_{max}}{d \cdot \alpha}, \tag{7}$$

$$P = {}^1/_3 \pi r^3 \frac{C}{100}, \tag{8}$$

where C is the concentration in %, d the diameter of the droplets in cm, α the refraction coefficient equal to 0.0018, P the absolutely dry weight of substances in the drop in g, $4/3\pi r^3$ the volume of the drop in cm^3, and δ_{max} the maximum wave path difference.

The maximum path difference between the beam which has traversed a spherical drop and one which has passed outside of it (through the medium surrounding the drop) will take place when the beams will go through the thickest part of the drop, i.e., along its diameter. If the droplets are fine, it is necessary to read the integrated wave path difference over the entire drop. This path difference is referred to as the average difference and comprises 2/3 of the maximum path difference. A suitable correction is introduced in calculating the concentration of the substance [817].

Then /82

$$C = {}^3/_2 \frac{\delta_{av}}{d \cdot \alpha}. \tag{9}$$

In addition, the weight of the substances in the drop can be calculated from the formula

$$P = \frac{S\delta_{av}}{100\alpha} = {}^2/_3 \frac{\delta_{max}}{100\alpha}, \tag{10}$$

where S is the area of the drop (area of the circle); the other symbols in the formulas (9-10) are the same as in formula (7-8).

Hence, in order to determine the concentration and weight of substances in drops, it is necessary to know the maximum or averaged path difference.

Measurements of the path difference δ were made on five models of interference microscope. Four of them were experimental models built by Zakhar'yevskiy, Gal'pern and Kuznetsova [154—155], and one was the "Nife" model of the Swedish company Junger [728—731, 920].

At the present time, all the existing interference microscopes can be divided into two types - bifocal and shearing microscopes.

The main difference between them lies in the fact that in the bifocal microscope the splitting of the interfering rays takes place along optical axis of the microscope, whereas in the shearing microscope the splitting takes place perpendicular to this axis. Therefore, in the shearing microscope a double image of a single object is obtained, whereas in the bifocal microscope a single image is obtained.

In our study, use was made of shearing microscopes with objectives ×3.7.0.11; ×40.0.65 and ×65.1.25 and with eyepieces ×10 and ×15, and also bifocal microscopes with objectives ×8, ×0.20 and ×40.0.65 and eyepieces ×15 and ×20. The use of differing microscope systems made it possible to check the results obtained with different instruments, and also to carry out measurements with coacervate drops 1 to 200 μ in diameter. Simplified diagrams of the shearing and bifocal type microscopes employed in the study are shown in Fig. 27. All the quantitative measurements were made in monochromatic light with a wavelength of 546 mμ; in the calculations the wavelenght taken was 0.55 μ.

Shearing Microscopes. Figure 27, a, shows a diagram of a shearing microscope with objective ×3.7.0.11. In this diagram, the rays from light source (J) pass through the light filter (Φ) and are polarized by a polaroid (P). Each beam of rays is then split in two by a Wallaston prism (W_1); two paris of rays are thus formed which pass through a condenser (K). One of the rays of each /83
pair then passes through the medium, and the other through the coacervate drop. Upon emerging from the preparation (B) containing the coacervate, the rays in the pair are no longer the same and have a definite phase difference. These rays pass through objective (O), are collected by the Wallaston prism (W_2), and arrive successively in the measuring device, which in the given model consists of the same Wallaston prism (W_2) and analyzer A. From the analyzer, having passed through the eyepiece, each pair of rays gives a single image of the drop in the field of view. Since there are two such pairs in our diagram, there will also be two images of the same drop (right and left) in the field of view.

When the drops are illuminated with white light, colored Newton rings are formed as a result of interference (Fig. 28, a, b). In monochromatic light, the alternation of light and dark rings is observed. The transition from one phase to the other proceeds through a series of intermediate steps dependent on the position of the Wallaston prism (W_2). In Fig. 29, a and c, two images of the same drop are in opposite phases, and in Fig. 29, b, in the same phase.

The process of measurement of the phase difference amounts to the following.

The instrument is adjusted for darkness of the field (background) by means of the analyzer. In this case, the drops remain light; by moving the Wallaston prism up or down, one and then the other image of the drop is brought to a given

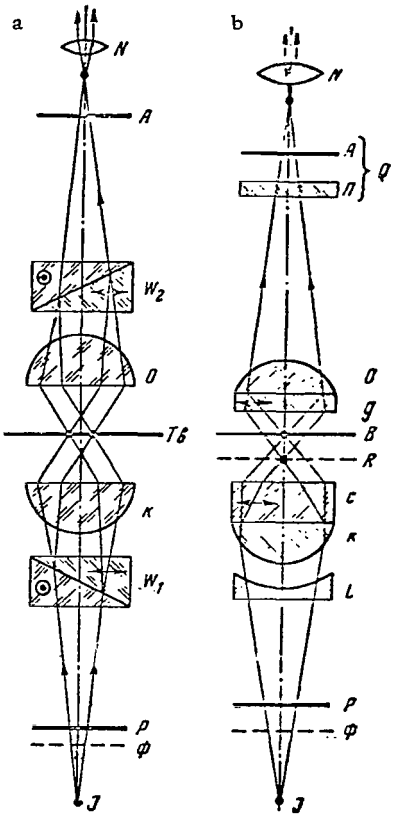

Figure 27. Diagrams of Interference Microscopes.

a—Shearing Type; b—Bifocal Microscope; J—Light Source; O—Objective; Φ—Light Filter; K—Condenser; C—Splitting Plate; B—Preparation and One of the Foci; R—Focus; g—Collecting Plate; Q—Senarmon Compensator; π—Quarter-wave Plate; A—Analyzer; L—Lens; W_1, W_2—Wollaston Prisms; N—Eyepiece; P—Polaroid.

phase, and two readings are taken, one for the first image and the other for the second. Usually, for large drops, the readings are taken along the diameter, when a dark circle is formed at the center of the drop; for fine drops, the reading is taken when the entire drop turns black. The reading is taken on a drum attached to the Wollaston prism (W_2). The drum has 100 divisions and a vernier to within 0.1. Twenty-two divisions of the drum correspond to a phase difference of 1 wavelength. The path difference is calculated from the formula

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$$\delta = \frac{K_r - K_\ell}{22 \cdot 2} \lambda, \qquad (11)$$

where K_r is the reading for the right image, K_1 is the reading for the left image, δ is the maximum or averaged path difference, 2 is a double image of the same drop, and λ is the wavelength, 10^{-4} cm.

The accuracy of the measurement $\delta = 1/50\lambda$. The microscope was used for large drops (objects). An example of the calculation follows.

Composition of the drop, RNA-gelatin-gum; diameter $d = 69 \cdot 10^{-4}$ cm, volume = 171.6 x 10^{-9} cm^3; K_r = 182; K_ℓ = 68.

$$\delta_{max} = \frac{(182-68) \cdot 0.55}{22 \cdot 2} = 1.425 \cdot 10^{-4} \text{ cm}$$

$$\text{Concentration } C = \frac{\delta_{max}}{d \cdot \alpha} = \frac{1.425 \cdot 10^{-4} \text{ cm}}{69 \cdot 10^{-4} \text{ cm} \cdot 0.0018} = 11\%,$$

where $\alpha = 0.0018$.

$$\text{Weight } P = 4/3 \pi r^3 \frac{C}{100} = \frac{171.6 \cdot 10^{-9} \cdot 11}{100} = 19.7 \cdot 10^{-9} \text{ g}.$$

Shearing Microscope With Objective ×65 · 1.25. The principal of the action of the "Nife" microscope of the Junger Company (1960 model) is the same as in the preceding model, but this model differs somewhat in design. A microscope has a Franson eyepiece with a measuring attachment. The Wollaston prisms W_1, W_2 are replaced by a Savart plate. Adjustment for darkness of the view is accomplished by means of this plate [565].

In contrast to the shearing model with objective ×3.7 the splitting of the image of the drop does not go to the left and right, but to the top and bottom. The reading of these phases in drops, as in the preceding microscope, is taken along a graduated circle mounted on a movable wedge (Q). The entire circle is divided into degrees, from 30 to 150°, and has a vernier permitting readings down to 0.1° The degrees are used to determine angle β, formed between the plates (F) when the Franson eyepiece is rotated. Usually, the measurements are made within a range of 90°. From the readings, angle β is found by use of the formula

$$\angle\beta = \frac{H - B}{2}, \tag{12}$$

where H is the reading of the lower image of the drop, B is the reading of the upper image of the drop, and 2 corresponds to the two images of the drop.

From angle β, sin β is found in trigonometric tables, then from the calibration curve on which sin β, is laid off along the ordinate axis, and the path difference in A is laid off along the abscissa axis, δ is obtained. The calibration curve was plotted by Johansson specifically for this instrument and is applied to the microscope.

This instrument makes it possible to study fine coacervate droplets. The accuracy of the measurement is 1/100 λ.

Example of Calculation.

Composition of drop, oleate-gelatin; diameter d = 10.36 x 10^{-4} cm; volume = 58.2 x 10^{-12} cm^3;

$$H = 112.6;\quad B = 64.6;\quad \angle\beta = \frac{112.6 - 64.6}{2} = 24°;\quad \sin\beta = 0.407;\quad \delta_{max} = 0.33\ 10^{-4}\ \text{cm}.$$

$$\text{Concentration } C = \left(\frac{\delta_{Max}}{d \cdot \alpha}\right) = \frac{0{,}33 \cdot 10^{-4}\ \text{cm}}{10{,}36 \cdot 10^{-4}\ \text{cm} \cdot 0{,}0018} = 18\%. \qquad /85$$

$$\text{Weight } P = 4/3\,\pi r^3 \frac{C}{100} = \frac{58 \cdot 2 \cdot 10^{-12} \cdot 18}{100} = 103 \cdot 10^{-12}$$

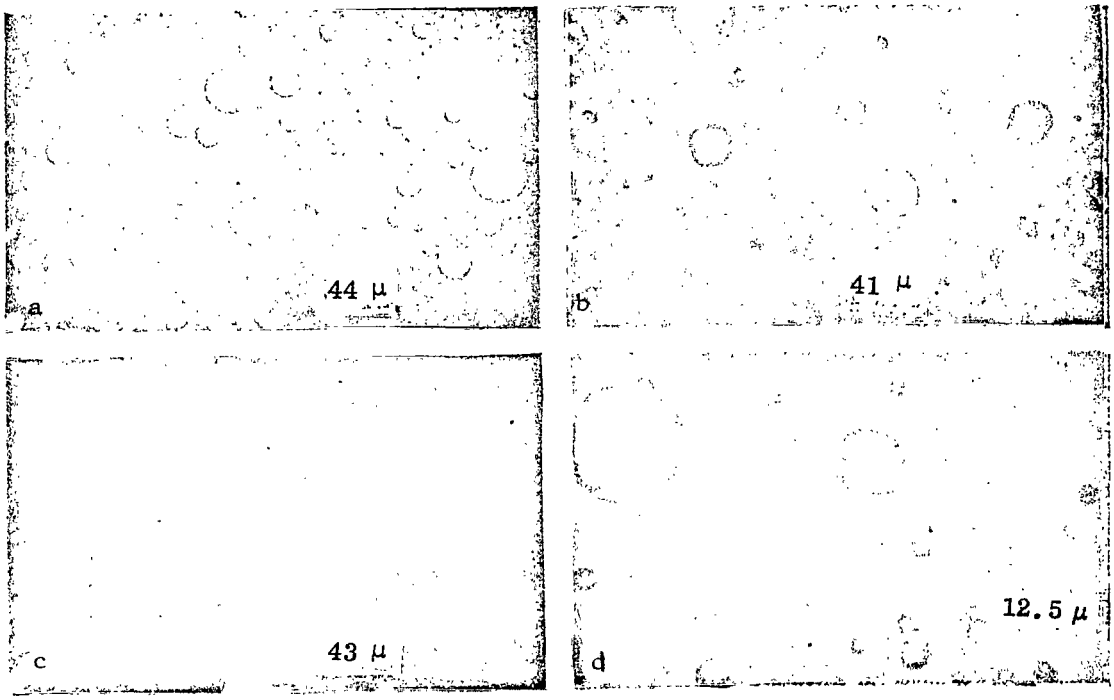

Figure 28. Appearance of Coacervate Drops.
a—Under Ordinary Microscope; b—Under Interference Microscope; c—Under Ultraviolet Microscope with a Luminescent Converter; d—Coacervate Drops Containing Chlorophyll (after Serebrovskaya).

A diagram of the bifocal microscope is shown in Fig. 27, b.

From the light source J, the rays passing through the light filter are polarized by polaroid P, and then pass through a negative quartz lens L and strike the front lens of the condenser (K) bonded to plate C, which splits the rays along the optical axis of the microscope and gives two foci at points of intersection K and B with two dark points. One pair of rays passes through the medium, and the other through the drop, and thus, upon emerging from the preparation (B), they have a certain phase difference. The rays are then recombined with plate G bonded to objective O. At the exit from the objective, the rays are directed into a Senarmon compensator (Q) consisting of a quarter-wave plate (*П*) an analyzer (A), and finally, after passing through the eyepiece, give a single image of the drop in the field of view. As in the shearing microscope, upon illumination with white light, the drop shows colored Newton's rings, and in monochromatic light, dark rings. A diagram of the phase shift in the drop is given in Fig. 30.

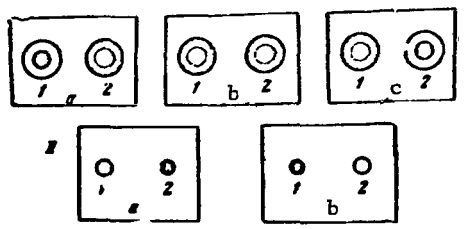

Figure 29. Phase Change in a Split Image of a Single Coacervate Drop Under a Shearing Type Interference Microscope.
I—Large Drop; II—Small Drop; a, c—Opposite Phases; b—Same Phase; 1—Left Image of the Same Drop; 2—Idem, Right Image.

For quantitative determinations, use is made of a reading attachment mounted on the analyzer. The graduated circle of this attachment is divided into 360° and has a vernier down to 0.1°. One wavelength corresponds to 100°.

The measurement of the phase difference consists in the following.

Adjustment for darkness of the field is made by crossing the analyzer with the polarizer at a 90° angle, and the reading is recorded. In this case, the drops appear light. The analyzer is then rotated so that the rays form a dark core (δ_{max}) at the center of the large drops, or unitl the entire fine drop (δ_{av}) darkens, and the second reading is recorded. Thus, the path difference is calculated from the formula /86

$$\delta = \frac{K_d - K_f}{180}\lambda, \qquad (13)$$

where K_d is the reading for the drop, K_f is the reading for the field or background, 180—1 λ. The accuracy of the measurement is 1/100 λ.

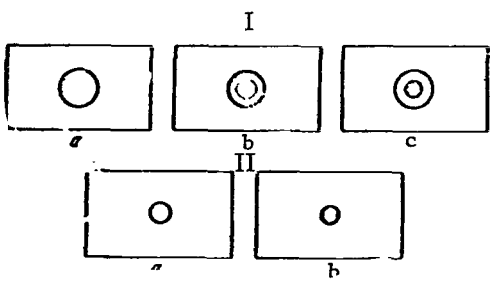

Figure 30. Phase Change in a Single Image of a Coacervate Drop Under a Bifocal Interference Microscope.
I—Large Drop; II—Small Drop; a-c—Successive Phase Change; c—Phase in Which Measurements are Made.

Example of Calculation.

Composition of drop, histone-RNA; diameter "d" = 3.96 x 10^{-4} cm; volume = 32.5 x 10^{-12} cm^3; K_d = 78; K_f = 1.

$$\delta_{av} = \frac{(78-1)\cdot 0.55\cdot 10^{-4}\ cm}{180} = 0.236\cdot 10^{-4}\ cm;$$

$$\text{Concentration } C = 3/2\,\frac{\delta_{av}}{d\cdot\alpha} =$$

$$3/2\,\frac{0.236\cdot 10^{-4}\ cm}{0.0018\cdot 3.96\cdot 10^{-4}\ cm} = 33\%.$$

$$\text{Weight } P = 4/3\,\pi r^3 \frac{C}{100} =$$

$$= \frac{32{,}5 \cdot 10^{-12} \cdot 33}{100} = 10{,}6 \cdot 10^{-2}\ \text{g}$$

$\alpha = 0.0018$.

The experimental model of a shearing with a $\times 40 \cdot 0.65$ objective differs some what from the shearing system with a $\times 3.7 \cdot 0.11$ objective

The Senarmon compensator is mounted after the eyepiece. The microscope gives two images of a single drop (object). The distance from one image to the other is 150 x (10^{-4} cm). One image is usually fairly pale and indistinct. For this reason, in measuring the phase difference, the reading is taken on the background field and on the distinct image.

The adjustment of the instrument for darkness of the field, drops and readings was carried out as for the bifocal microscope $\times 40 \cdot 0.65$. The advantage of the given model over the bifocal microscope $\times 40$ is the absence of a reference region and a more accurate reading to 1/100—1/150 λ.

Example of Calculation. /87

Composition of drop, phosphorylase – histone – gum – starch and other compounds.

Diameter, 2.5×10^{-4} cm; volume, 8.36×10^{-12} cm^3.

Distinct single image of drop at $K_d = 100$ and $K_f = 45$;

$$\delta_{av} = \frac{(100-45) \cdot 0{,}55 \cdot 10^{-4}\ \text{cm}}{150} = 0{,}165 \cdot 10^{-}\ \text{cm}.$$

$$\text{Concentration } C = 3/2 \frac{\delta_{av}}{d \cdot \alpha} = 3/2 \frac{0{,}165 \cdot 10^{-4}\ \text{cm}}{2{,}5 \cdot 10^{-4}\ \text{cm} \cdot 0{,}0018} = 54\%.$$

$$\text{Weight } P = 4/3\,\pi r^3 \frac{C}{100} = 4{,}62 \cdot 10^{-12}\ \text{g}.$$

If the path difference caused by the drop (object) is greater than 1 λ., in bifocal and shearing type microscopes a compensator is introduced which gives interference bands in the field of view. In this case, measurements of several λ can be made. Usually, whole values of λ are read off by means of such compensators (Babine et al.). Fractions of waves are more accurately measured with

the Senarmon compensator. Babine type compensators are convenient for determining the weight in an object with an uneven distribution of substances.

Interference Microscopes With Bands in the Field of View. In our experiments, such bands were obtained by introducing a Wollaston prism into the optical system of interference microscopes. On illumination of the field of view with white light, the bands were colored and resembled a rainbow, and were dark in monochromatic light.

Figure 31 shows diagrams of the band shift in a spherical object after Davies (644) and in a coacervate drop. Figure 32 shows a photograph of such a drop under the microscope. The figures showed that the greatest band shift takes place along the diameters of the sphere or drop.

Measurement of the maximum path difference from the shift of the interference bands is made by using the formula [14]:

$$\delta = \frac{b}{a}\lambda, \qquad (14)$$

where a is a segment corresponding to one wavelength (distance between bands); b is the shift of the band along the diameter of the drop; λ is the wavelength.

Obviously, for the same diameter of the drops, the shift will be greater the higher the concentration of the substances in the drop. The numbers of the interference bands increase in the direction of the arrow. If the band in the drop is bent toward a smaller number, the path difference δ is positive and the concentration of the substance of the drop is greater than in the equilibrium liquid. If the band is distorted along the direction of the higher number, δ is negative, and the concentration of the substance of the drop should be less than in the liquid surrounding it (we did not observe such a case). /90

In numbering the bands, use is made of a standard giving a positive path difference.

From the bending of the bands one can form an idea of the concentration of the compounds not only in objects with a homogeneous distribution of substances, but also in structured drops and cells. If an inhomogeneous localization of the dry mass is observed in individual portions of the drop or cell, the bending of the band will change correspondingly. As an example, Fig. 32-34 show photographs of a coacervate drop with a vacuole and photographs of an erythrocyte. In both cases, a change in the direction of the bending of the bands takes place in the center, indicating a decrease of the dry matter content.

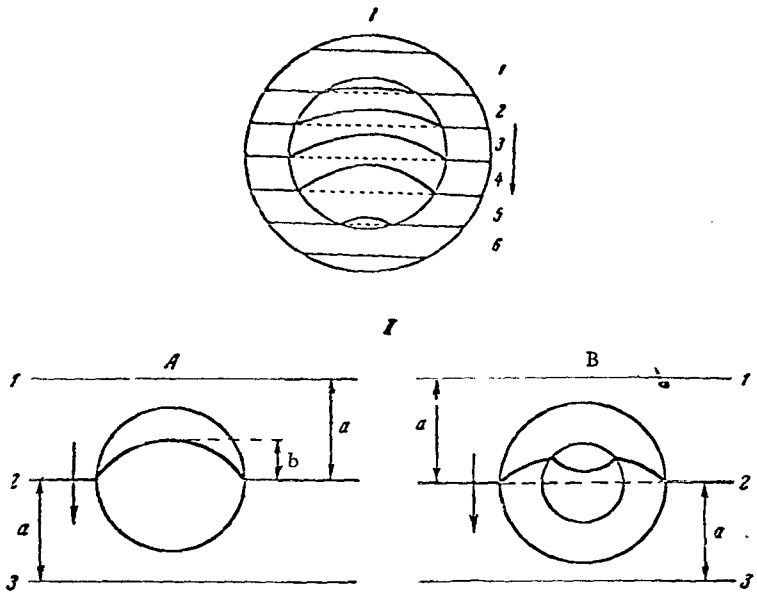

Figure 31. Diagram of Image of Objects Under Interference Microscope with Bands in the Field of View.

I—Globular Object (after Davies); II—Coacervate Drop; A—Homogeneous Drop; B—Drop with Vacuole; a—Distance from one Interference Band to the Next; b—Distance from the Top of the Band Shift to the Start of its Entrance into the Drop; Arrows—Direction of Light of Interference Bands (1-7).

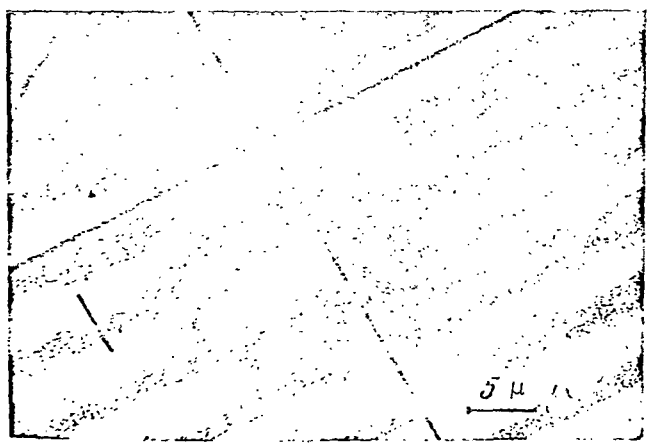

Figure 32. Appearance of Coacervate Drop Under Interference Microscope with Bands in the Field of View.

Arrow—Direction of Counting of the Bands.

In the erythrocyte, this is the result of flattening, the edges being thicker than the center, and in the drop this effect produces a vacuole.

As follows from the diagram and photographs (see Fig. 32, c), the bending of the bands in the sphere surrounding the vacuole is greater than in the vacuole itself. Hence, the concentration of substances in the vacuole is lower than in the drop.

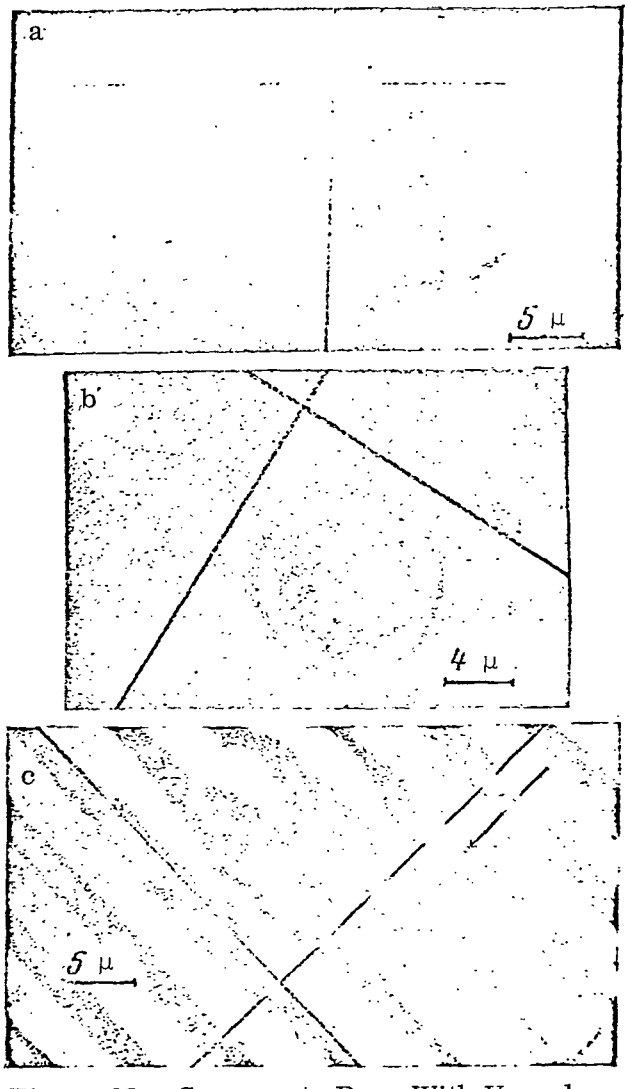

Figure 33. Coacervate Drop With Vacuole. a—Negative Contrast; b—Positive Contrast; c—Under Interference Microscope with Bands in the Field of View; Arrow—Direction of Counting of Bands.

Tentative calculations of the concentration of substances in the vacuoles and in the sphere surrounding it were carried out by measuring the maximum path difference introduced by the sphere and vacuole.

If one considers that in the sphere surrounding the vacuole the distribution of substances is homogeneous, the concentration of substances in this sphere will be the same everywhere. At the same time, the height of the bend /91 of the interference band will depend on the part of the sphere traversed by the rays. If the vacuole is located at the center of the drop, the greatest height of the bend of the band will be observed at the boundary with the vacuole. The corresponding diameter of the conventional drop can be taken as the distance from the edge of the drop to the vacuole. In this case, the concentration in the sphere can be calculated from the following formula:

$$C_{\text{sphere}} = \frac{h_{\text{sph}} \cdot 0{,}55 \cdot 10^{-4}}{A \cdot \alpha \cdot \delta_{\text{sph}}}, \quad (15)$$

where h_{sph} is the height of the bend of the band in the sphere in in cm, A is the height in cm = 1 λ; λ = 0,55 · 10^{-4} cm = wavelength of light, α = 0.0018, and δ_{sph} is the distance from the edge of the drop to the boundary with the vacuole.

Concentration of Substances in Vacuole+Sphere. In order to determine the concentration of substances in the vacuole, the height of the bend of the band in the center of the vacuole is measured. This height is usually lower

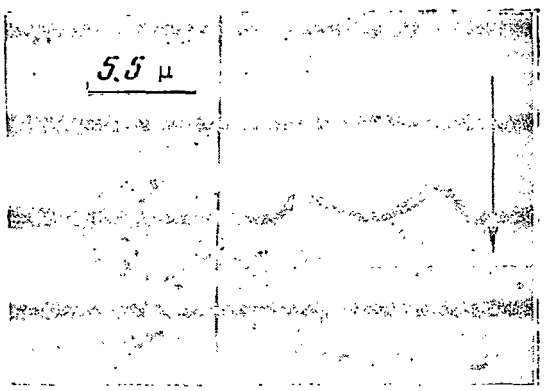

Figure 34. Appearance of Erythrocyte Under Interference Microscope With Bands in the Field of View (photo by A. F. Kuznetsova). The Bend of the Band Indicates Flatness of the Erythrocyte at the Center.

than at the edges of the vacuole. This is due to the fact that the vacuole is surrounded by a sphere in which the concentration of substances is much higher than in the vacuole. Before reaching the vacuole, the rays pass through the sphere surrounding it. The thinner the sphere, the smaller the bend of the band. The concentration of substances in the vacuole was calculated from the formula

$$C_v = \frac{h_v \cdot 0.55 \cdot 10^{-4}}{A \cdot \alpha \cdot D}, \qquad (16)$$

where C_V is the height of the bend along the vacuole diameter in cm and D is the vacuole diameter. The remaining symbols are the same as in formula (15).

The concentration obtained was found to be higher than that which should prevail in vacuoles, since the bend of the band in the vacuole depends on the concentration of substances not only in the vacuole but also in the sphere. When this method is used, the calculations of the diameter of the drops and vacuoles and also path differences can be carried out by using photographs.

This is rather a labor-consuming process, since usually in the field of view with a ×40 objective, no more than 4 to 5 drops can be measured on a single photograph. It is impossible to arrange all the drops so that the bend of the band runs along the diameter of the drop (Table 17)

TABLE 17. Content of Substances in Drops With Vacuoles in a Gelatin-Gum-DNA Coacervate

Diameter, 10^{-4} cm		Volume, 10^{-12} cm^3		Concentration, %	
Drop	Vacuole	Drop	Vacuole	Sphere	Vacuole + sphere
11.07	6.07	703.7	116.0	14	6
15.35	9.64	1876.2	464.7	11	9
5.35	2.14	79.4	5.0	24	10
8.12	4.28	368.2	40.7	21	10
7.14	3.93	188.72	31.5	17	12
11.78	5.0	848.01	64.8	18	13

It follows from the data of Table 17 that the concentration found in the vacuole is the sum of the true concentration of the vacuole and the sphere surrounding it and is considerably below the concentration of substances in the sphere. Obviously, the concentration in the vacuoles themselves will be several times less than the values indicated for the concentration of the vacuole and the sphere. In some cases, when the vacuole occupies a considerable portion of the volume of the drop, the bends of the interference bands almost coincide with the straight interference band, i.e., the concentration in the vacuoles corresponds to the concentration in the liquid surrounding the drop and amounts to hundredths of one per cent. /92

Our data are preliminary. A more exact determination of the concentration in vacuoles will be possible if somewhat different methods are used for measuring the path difference, in particular, by using the curve of the distribution of the path difference in scanning microscopes [921].

A characteristic feature of all interference microscopes is that the shape of the drops observed under the microscopes is independent of the chemical nature of the substance. Therefore, the illustrated photographs of coacervate drops will be the same for drops obtained from components which are most diverse in chemical composition.

It should be noted that shearing microscopes give a sharper image [826].

Errors of the Method of Interference Microscopy. Errors in the determination of the content of dry substances in cells or particles by means of interference microscopy amount to 0.1-10% of the value being determined.

Such fluctuations depend on the specimen itself as well as on the design of the instrument [622, 639, 640, 643, 705, 707, 873—874].

The following formula was used for calculating the errors in determinations of the dry weight of substances in the drops:

$$P = \frac{S\delta_{\text{av.}}}{100\alpha} = 2/3\frac{S\delta_{\text{Max}}}{100\cdot\alpha}, \qquad (17)$$

where P is the weight in g, S is the area of the circle $= \frac{\pi d^2}{4}$; δ is the path difference in cm and d is the diameter of the drop in cm.

From formula (17) it follows that the errors may depend on two factors: 1) on the measurement of the size of the drop, in this case the diameter, and 2) on the measurement of δ, the path difference.

The first error occurs in all microscopic investigations involving the determination of the size of the object. The second error depends on the accuracy of the measurement of the path difference in the interference microscope.

Both errors are calculated as corresponding derivatives of formula (17).

The first derivative, which gives an idea of the maximum error in the result of measurement of the path difference and is denoted by P_1, is calculated from the formula /93

$$P_1 = \frac{\pi d^2 \Delta\delta}{100 \cdot 4\alpha}, \tag{18}$$

where $\Delta\delta$ is the limiting accuracy of the measurement of the path difference in the given microscope.

The second derivative, denoted by P_2, indicates the error obtained as a result of measuring the diameter, and is given by the formula

$$P_2 = \frac{\delta\pi 2d\Delta d}{100 \cdot 4\alpha}, \tag{19}$$

where Δd is the accuracy of the reading of the drop diameter.

The absolute and relative errors were calculated from the formulas

$$P_{abs.} = \pm \sqrt{(P_1)^2 + (P_2)^2}, \tag{20}$$

$$P_{rel.\,\%} = \frac{P_{abs} \cdot 100}{P}, \tag{21}$$

where P is the weight of the drop, P_{abs} is the absolute error, and P_{rel} is the relative error.

The average relative error in the determination of the dry weight of drops for all the coacervate systems studied amounts to 6.2% of the value being determined.

In each system, the greatest relative error will take place for fine drops. The finest drops, less than 1 μ in diameter, were not considered, since they are beyond the scope of the instruments employed, and the errors in this case may be above 26%.

The relative error as a function of the diameter of the drops is listed below:

Diameter, 10^{-4} cm	Mean relative error, %
1.5-5	8.5
5-10	7.2
10-30	4.45
30-100	1.9

Thus, the lowest relative error is associated with the largest drops.

Results of comparative determinations of the content and concentrations of substances in the same drops but under different microscopes are listed in Table 18.

TABLE 18. Determination of the Content of Substances in Drops /94

Model of Interference microscope	Composition	Coacervate Drop			
		Diameter 10^{-4} cm	Volume 10^{-9} cm	Weight 10^{-9} g	Concentration, %
Shearing ×3.7	Gelatin-gum	32.2	17.44	4.4	23.65
		34.5	21.4	4.38	20.45
		34.5	21.4	4.34	20.28
Bifocal ×8		32.2	17.44	4.06	23.27
		34.5	21.4	4.29	20.06
		34.5	21.4	4.28	20.00
Shearing ×65	Gelatin-potassium oleate				
Shearing ×40		20.02	4.2	0.73	17.48
Bifocal ×40		20.02	4.2	0.74	17.81

From the data of Table 18 it follows that the discrepancies between the individual measurements of concentrations in different models of microscopes for the same drops amounted to approximately 0.5%.

This value is not a large error, since the concentrations of tne dry matter are usually calculated in whole per cent numbers, and tenths are not considered.

On the average, in each coacervate system 100 drops from different samples with two or three repetitions were measured. Many drops having

equal diameters were observed. In this case, particularly within the confines of a single sample, such drops usually had the same weight and concentration.

Relationship Between the Weight, Volume and Concentration of Substances in Drops and Cells

Of greatest interest are data on the concentration of substances and dry matter in individual drops differeing in size and chemical conposition. This is important for elucidating the characteristics of coacervate systems and comparing them with data obtained for cells along these lines.

The weight of the dry matter and the concentration of substances in drops were determined chiefly in two-component coacervate systems whose composition included proteins, neucleic acids, carbohydrates and lipids.

The systems were selected so that only one component would change, and the second remain constant. For instance, in protein-carbohydrate drops, different proteins were combined with a single carbohydrate. The same applies to coacervates of proteins, i.e., proteins where histone and gelatin were studied in combination with serum albumin and clupein. In protein-nucleic systems, the same protein gave coacervates with RNA and DNA. Conversely, RNA or DNA coacervated with different proteins.

Such combinations were taken in order to be able to determine more readily the characteristics of each system as a function of the change of one of the components. In addition, a multicomponent coacervate with the participation of an enzyme was prepared. The compositions of these coacervates are shown in more detail in Table 11.

These systems contained drops of various sizes which has a volume of $n \times 10^{-12}$ cm^3 and also $n \times 10^{-9}$ cm^3.

The characteristics of a majority of coacervate systems as a function of the number of drops and their size are given in Fig. 35-38 [153]. Such measurements were carried out with a microspecimen analyzer in the radio electronics laboratory of the Biophysics Institute of the USSR Academy of Sciences.* In this instrument, the number of cells (coacervate drops) or other microspecimens of different diameters is determined automatically in the field of view of the microscope [134—135]. In each coacervate, an average of 1000 drops were counted.

* We take this opportunity to thank G. N. Orlovskiy and L. L. Litinskaya for their consultations and Acad. G. M. Frank and L. B. Kaminir for affording us the opportunity to work at the Institute.

As follows from Fig. 35 (A-D), coacervate systems in which fine droplets predominate are formed from compounds whose isoelectric points are the most widely separated from each other. For example, they include droplets from alkaline proteins + nucleic acids.

At the same time, coacervates containing substances with closer isoelectric points, for example gelatin and gum arabic, have larger drops. In these systems, single drops several tens of microns and even over 100 mμ in diameter are also present. The number of such drops is very small, and frequently amounts to less than 1% of the total amount of all the drops, and therefore is not shown on the graphs.

Figures 36-40 show the changes in the weight of the dry matter of the drops as a function of their volume. The broken line denotes the course of the change in the calculated weight of the drops at a constant concentration in the drops independently of their size. The value found in the smallest droplet was taken as the constant concentration. The solid line indicates the course of the change in the weight of each drop, obtained experimentally. In order to have an idea of the concentration of substances in any drop, it is sufficient to divide its dry weight by its volume.

These graphs are typical of all the coacervate systems listed in Table 11.

From Figs. 36-40 it follows that the discrepancy between the experimental and calculated results increases with the size of the drops. The volume of the drops increases faster than their dry weight. This lag of the weight behind the volume is associated with a decrease of the concentration of substances in the drops in all 16 systems. The absolute concentration values may change with the chemical composition. Cumulative Table 19 lists absolute values of the concentrations and dry matter of drops of similar size but of different chemical composition. It should be noted that in order to obtain coacervate systems, the frequency of the chemical preparations employed, particularly nucleic acids is of great importance. /97

The coacervates listed in Table 19 have several characteristics which distinguish them from each other.

1. Coacervates consisting of different proteins in combination with a carbohydrate-gum and also systems of alkaline-acid protein contain various drops in which the concentrations of substances vary from 8 to 72%.

2. In the histone-serum albumin coacervate, an interesting phenomenon was observed. The small droplets which appeared became larger and frequently dissolved. The growth was due chiefly to swelling with water and was associated with a strong decrease of concentration. A very representative droplet had a volume of 4.0×10^{-12} cm^3 (diameter 1.98×10^{-4} cm) and a concentration

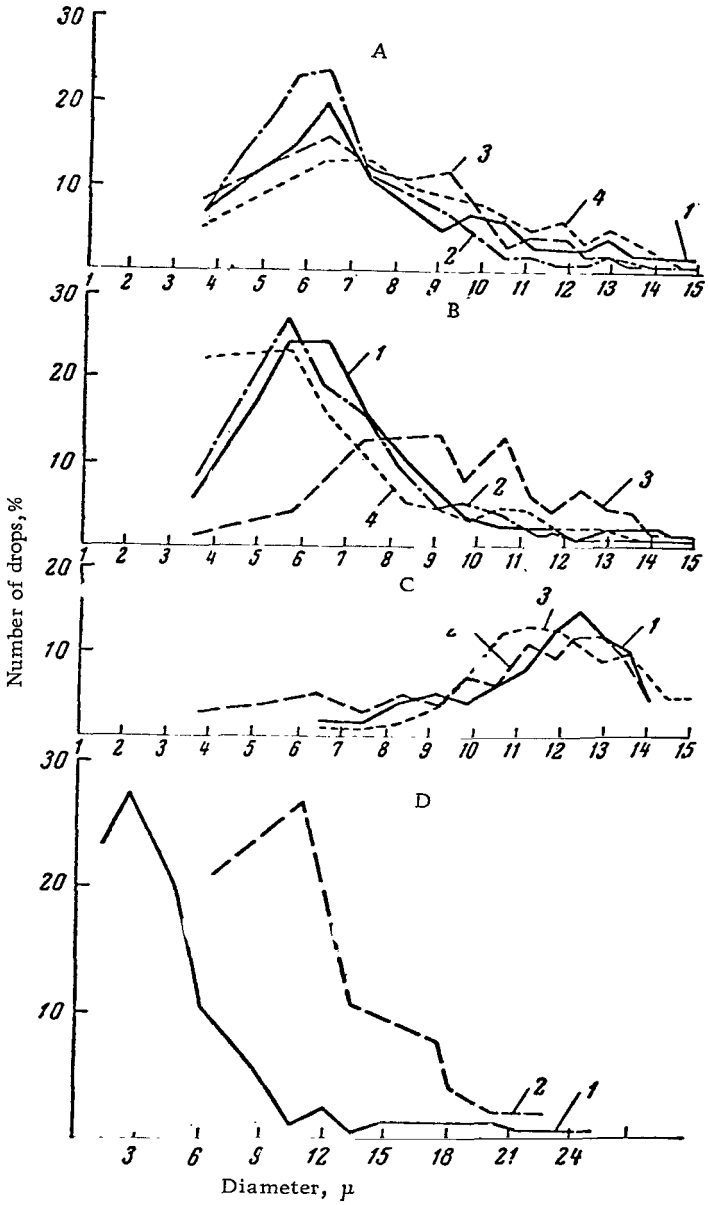

Figure 35. Size Distribution of Drops in Coacervates (the Total Number of Drops of Various Sizes is Taken as 100%).

A: 1—Clupein - DNA; 2—Clupein - RNA; 3—Histone - DNA; 4—Histone - Gelatin; B: 1—Clupein - Gelatin; 2—Histone - DNA; 3—Histone - Serum Albumin; 4—Sickle Protamine - Gum. C: 1—Gelatin - Gum - RNA; 3—Gelatin - Gum - DNA; D: 1—Multicomponent Coacervate of Histone - Phosphorylase, etc.; 2—Potassium Oleate - Gelatin.

of substances of 85% (weight of dry matter 3.4×10^{-12} g). In 2-3 sec, the drop increased by a factor 37.5, the volume became $150 \times 2 \times 10^{-12}$ cm^3, the diameter 6.6×10^{-4} cm^3, the weight 13.9×10^{-12} g, and the concentration decreased to 9%. Such a process usually lasts a short time, and the remaining drops do not undergo any further changes. The solution of drops was first observed by Bungenberg de Jong, who explained this phenomenon by the denaturization of syrup albumin [593]. Obviously, other causes also exist, since part of the drops are more stable. It is possible that drops having an excessively high concentration soak up the solution too rapidly, swell up and cannot withstand the force of gravity and hydration acting so as to crush the drops.

However, a complete explanation of this phenomenon requires additional data.

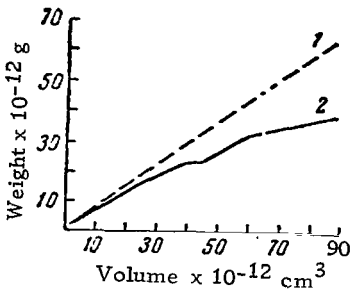

Figure 36. Coacervate Drops of Clupein-DNA.

1—Weight of Drops Calculated at a Constant Concentration of Substances, Measured in the Drop of the Smallest Diameter; 2—Weight of Drops Obtained Experimentally.

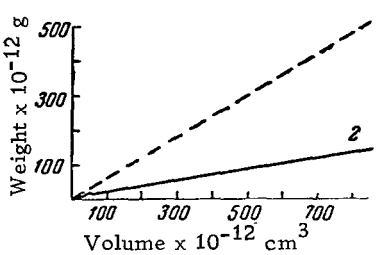

Figure 39. Coacervate Drops of Serum Albumin-Histone.

Notation same as in Fig. 36.

The remaining coacervate systems contained stable drops.

It is necessary to emphasize that nucleic acids+alkaline proteins form dense, fine droplets, and that in coacervates containing clupein, the greatest concentration of molecules is observed in the drops.

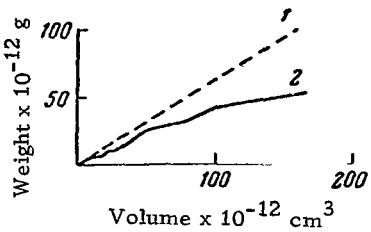

Figure 37. Coacervate Drops of the Multicomponent Coacervate Phosphorylase - Histone - Starch.

Notation same as in Fig. 36.

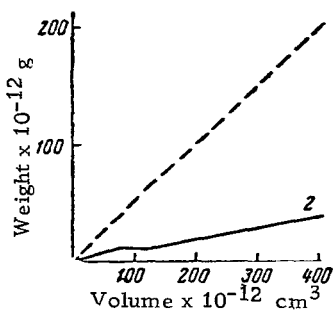

Figure 38. Coacervates Drops of Histone - DNA.

Notation same as in Fig. 36.

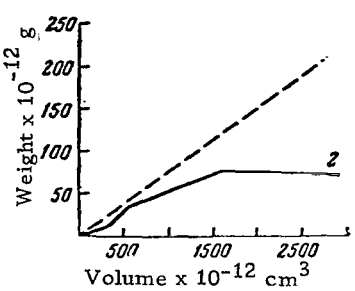

Figure 40. Coacervate Drops of Gelatin-Gum Arabic.

Notation same as in Fig. 36.

TABLE 19. Total Content of Dry Substances in Coacervate Drops /99

No.	Diameter, 10^{-4} cm3	Volume, 10^{-12} cm3	Weight, 10^{-12} g	Concentration, %	$C_{drop}/C_{solution}$*	Composition**
			Protein-Carbohydrate			
			Serum albumin-gum (p^H 4.4)			
1	2.2	5.5	2.9	53	78	0.69% solution of serum albumin+ 0.67% solution of gum arabic (1:1), prepared with 0.1 M acetate buffer at pH 4.4 and 16-20°; C of total solution, 0.68%
2	3.08	15.3	5.3	34	50	
3	3.30	18.8	6.0	32	47	
4	6.16	122	25.6	21	31	
5	8.14	279.8	25.1	9	13	
			Histone-gum (pH 5.5-6.0)			
6	1.98	4.0	2.9	72	131	0.2 ml of 0.5% solution of histone +0.1 ml of 0.67% solution of gum arabic, pH 5.5-6.0 at 16-20°; C of total solutions 0.55%
7	3.30	18.8	9.8	52	94	
8	6.16	122.1	53.0	43	79	
9	8.36	305.3	103.8	34	61	
10	9.90	507.0	182.5	36	65	
			Sickle protamine-gum (pH 6.0-6.0)			
11	2.42	7.4	2.8	38	47	0.4 ml of 1.5% solution of sickle protamine sulfate+ 1.6 ml of 0.67% solution of gum arabic, pH 6.0 at 20°; C of total solutions 0.8%
12	4.40	44.6	13.8	31	39	
13	4.62	51.5	15.5	30	37	
14	6.16	122	32.9	27	33	
15	8.36	303.1	36.4	12	15	
			Starred sturgeon protamine-gum (pH 5.8-6.0)			
16	3.52	22.8	11.4	50	58	0.1 ml of 2% solution of starred sturgeon protamine sulfate + 0.6 ml of 0.67% solution of gum arabic, pH 5.8-6.0 at 16-18°; C of total solutions 0.86%
17	4.51	47.6	20.4	43	50	
18	6.26	127.9	43.4	34	39	
19	7.48	218.6	68.4	29	33	
20	7.92	257.3	66.8	26	30	
			Clupein-gum (pH 6.0-6.5)			
21	2.64	9.6	3.4	36	46	0.1 ml of 2% solution of clupein sulfate+1 ml of 0.67% solution of gum arabic pH6.0 at 16-18° C of total solution 0.79%
22	2.97	13.6	4.5	33	42	
23	3.19	16.8	5.4	32	41	
24	4.18	37.9	11.1	29	37	
25	6.27	128.2	35.9	28	36	

TABLE 19. Total Content of Dry Substances in Coacervate Drops (continued)

No.	Diameter, 10^{-4} cm^3	Volume, 10^{-12} cm^3	Weight, 10^{-2} g	Concentration, %	$C_{drop}/C_{solution}$*	Composition**
			Protein-Protein			
			Histone-serum albumin (pH 5.5-6.0)			
26	1.32	1.2	0.7	58	58	0.1 ml of 1% solution of histone + 0.5 ml of 1% solution of serum albumin, pH 5.5-6.0 at 10°; of total solution 1%
27	2.20	5.6	2.5	44	44	
28	3.96	32.4	11.3	35	35	
29	5.50	86.9	15.6	18	18	
30	9.02	383.4	65.2	17	17	

TABLE 19. Total Content of Dry Substances in Coacervate Drops

No.	Diameter, 10^{-4} cm^3	Volume, 10^{-12} cm^3	Weight 10^{-12} g	Concentration%	$C_{drop}/C_{solution}$*	Composition **
			Histone-gelatin (pH 6.2-7.3)			
31	2.20	5.6	3.2	57	96	0.2 ml of 0.5% solution of histone + 0.25 ml of 0.67% solution of gelatin pH 6.2-7.3 at 40°; C of total solution 1.2%
32	4.18	38.2	13.4	35	60	
33	5.50	86.9	26.1	30	50	
34	11.66	726.2	108.9	15	25	
35	11.88	876	87.6	10	17	
			Clupein-gelatin (pH 8.6-8.8)			
36	2.64	9.5	3.8	40	33	1.72% solution of clupein sulfate + 0.67% solution of gelatin (1:1), pH 8.6-8.8 at 40-45°; C of total solution 0.59%
37	3.41	20.5	7.0	34	28	
38	5.28	76.4	14.5	19	16	
39	5.94	108.7	13.0	12	10	
40	7.92	259.6	20.8	8	7	
			Protein-Nucleic Acid			
			Histone-RNA (pH 7.0-7.6)			
41	1.54	1.9	1.3	70	139	1 ml of 0.5% solution of histone + 0.2 ml of 0.5% solution of RNA, pH 7.0-7.6 at 16-20°; C of total solution 0.5%
42	1.76	2.8	1.6	58	116	
43	4.18	38.3	5.7	15	29	
44	7.92	412.9	45.4	11	24	
45	15.84	2080.1	83.2	4	8	
			Histone-DNA (pH 7.0-8.2)			
46	1.87	3.4	2.3	69	139	1 ml of 0.5% solution of histone + 0.1 ml of 0.5% solution of Na salt of DNA, pH 7.8-8.2 at 16-20°; C of total solution 0.5%
47	2.86	12.2	5.8	48	95	
48	3.08	15.3	7.2	47	94	
49	4.4	44.6	11.1	25	51	
50	16.72	2446.5	171.2	7	14	

/100

TABLE 19. Total Content of Dry Substances in Coacervate Drops (continued)

No.	Diameter, 10^{-4} cm^3	Volume, 10^{-12} cm^3	Weight, 10^{-12} g	Concentration%	$C_{drop}/C_{solution}$*	Composition**
		Clupein-RNA (pH 8.0-8.6)				
51	1.54	1.9	1.4	74	435	0.5 ml of 0.1% solution of clupein sulfate + 0.1 ml of 0.5% solution of RNA, pH 7.6-8.2 at 1-20°; C of total solution 0.17%
52	1.98	4.1	2.4	59	341	
53	3.3	18.8	8.6	46	269	
54	4.84	59.3	26.1	44	258	
55	7.15	191.3	59.3	31	179	
		Clupein-DNA (pH 7.6-8.2)				
56	1.76	2.8	2.3	79	566	1 ml of 0.10% solution of clupein sulfate + 0.1 ml of 0.5% solution of Na salt of DNA, pH 7.6-8.2 at 16-20°; C total solution 0.14%
57	2.2	5.6	3.4	61	436	
58	4.84	59.3	27.5	46	331	
59	5.28	77	26.3	54	244	
60	9.24	412.9	56.2	14	100	

TABLE 19. Total Content of Dry Substances in Coacervate Drops

No.	Diameter, 10^{-4} cm^3	Volume, 10^{-12} cm^3	Weight, 10^{-12} g	Concentration%	$C_{drop}/C_{solution}$*	Composition**
		Protein-Carbohydrate-Nucleic Acid				
		Gelatin-gum-DNA (pH 3.8-4.0)				
61	2.14	5.1	2.5	50	68	0.67% solution gelatin + gum arabic (5:3) + Na salt of DNA (0.60% DNA in the mixture), pH 3.8-4.0 at 40°; C of total solution 0.73%
62	2.32	6.5	2.4	37	51	
63	4.46	46.0	12.0	27	37	
64	5.35	79.4	19.8	25	34	
65	6.43	137.9	31.7	23	31	
66	34.5	21,400	4,900	24	33	
67	41.4	37,100	5,900	16	22	
68	62.1	125,100	12,510	10	14	
		Gelatin-gum-RNA (pH 3.8-4.0)				
69	34.5	21,400	42,800	20	28	0.67% solution of gelatin + gum arabic (5:3) + RNA (0.045% RNA in mixture), pH 3.8-4.0 at 40°; C of total solution 0.72%
70	53.8	81,300	11,380	14	19	
71	82.8	296,600	23,728	8	11	
		Protein-Carbohydrate				
		Gelatin-gum (pH 3.8-4.0)				
72	29.9	14,000	1,120	8	12	0.67% solution of gelatin + gum arabic (5:3), pH 3.8-4.0 at 40°; C of total solution 0.67%
73	85.1	322,000	19,320	6	9	
74	163.3	2275,300	22,753	1	1	

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TABLE 19. Total Content of Dry Substances in Coacervate Drops (continued)

No.	Diameter, 10^{-4} cm^3	Volume, 10^{-12} cm^3	Weight, 10^{-12} g	Concentration%	C_{drop} / $C_{solution}$*	Composition**
	Phosphorylase-histone-starch-gum (pH 6.0-6.2)					
75	1.32	1.2	0.8	69	103	Starch + NaF + acetate buffer + gum arabic + phosphorylase + glucose-phosphate + histone, pH 6.0-6.2 at 16–25°; C of total solution 0.67% (for method of preparation see Chapter 8)
76	2.42	7.4	3.7	50	75	
77	4.18	38.2	14.1	37	55	
78	5.50	86.9	24.3	28	41	
79	7.04	182.3	46.0	25	38	
	Gelatin-potassium oleate (pH 8.4-8.6)					
80	7.7	233.9	55.0	23	3.3	0.4 ml of 3% solution of gelatin + 2 ml of 1 M phosphate buffer, pH 8.4-8.6 + 0.6 ml of 0.1 N solution of K oleate at 40°; C of total solution 6.4%
81	14.84	1710.4	359.2	21	3.2	
82	16.94	2544.0	483.3	19	2.9	
83	21.56	5246.3	892.4	17	2.4	
84	24.36	7565.9	1135	15	2.4	

*$C_{solution}$ total concentration of substances in the initial solutions.

**Separated figures - extreme values obtained experimentally. Fine droplets were not measured in the systems gelatin-gum-RNA and gelatin-gum. In order to obtain the desired pH of the coacervate when there is no buffer present, the solutions were alkalized with 0.1 N NaOH or acidified with a 4% CH_3COOH solution.

The ratio /102

$$\frac{\text{concentration of substances of drop}}{\text{concentration of solutions}} = n \times 100$$

The highest increase of concentration, by a factor of 566, was noted for a drop of clupein-DNA.

For clupein-RNA and histone-RNA, about 72-82% of the dry mass was found in drops with a diameter of about 2 μ [129].

The picture changes if the same nucleic acids are present in combination with an acidic protein and a carbohydrate. These systems contain not only fine but also large drops. However, even in fine droplets (see Table 19), the absolute concentrations of substances are lower than in coacervates with alkaline proteins. The lowest concentrations of substances were found in the largest drops of gelatin-gum, which, owing to their large size, are the heaviest. The systems considered belong to two-component coacervates.

The multicomponent system taken was a coacervate whose composition included high and low molecular carbohydrates, alkaline and acidic proteins - the enzyme phosphorylase, and also mineral salts. The synthesis of starch was carried out in such a coacervate.

However, these drops did not differ from those of two-component coacervate systems.

A characteristic feature of a liquid-protein coacervate of gelatin-K oleate is the low degree of concentration of molecules in the drops as compared to the original solutions.

Protein, protein-nucleic, protein-carbohydrate, protein-lipid and multicomponent coacervate drops with the participation of enzymes are characterized by the following general properties:

1. The molecules of chemical compounds collect from the solutions in the drops, and this leaves almost no substances in the aqueous solution. A particularly high increase of concentration as compared to the original solutions (by a factor of hundreds) is observed in coacervate drops consisting of alkaline proteins and nucleic acids; this can be explained by marked differences in their isoelectric points. Each coacervate system contains drops having the most diverse concentrations of substances, although they were formed from the same solutions having the same concentration.

2. There is an inverse proportion between the size of the drop and its concentration of molecules. The larger the drops, the lower their concentration of various compounds. By analogy, such a phenomenon can be partly accounted for by Laplace's law concerning the dependence of the radius of curvature and pressure in capillaries [203]. The smaller the radius of the drop, the larger the compressive force expelling mainly water out of the drop and thus increasing the concentration of other substances [714]. /103

3. As a rule, as the diameter and hence the volume of the drops increases, their dry weight increases. However, exceptions exist. For instance, in the

gelatin-gum system, the largest drop, 177.1 μ diameter, weighs less than the next to the largest drop, having a diameter of 144.9 μ (the weight of these drops is respectively 74.6×10^{-9} and 77.9×10^{-9} g). Obviously, the growth of the drop is due to the absorption of water. In coacervate drops with vacuoles, the concentration of substances in the vacuoles is much less than in the sphere surrounding them.

4. Among the multitude of coacervate drops there are those whose size, weight and concentration are characterized by values close to unicellular organisms. For example, the weight of the bacterial cell B. lichenformus Ford. is equal to 1.84×10^{-12} g, and its length is 1.8 μ [133]. Highly suitable from the standpoint of these indices are drops of clupein-DNA, 1.87 μ in diameter, having a weight of 1.87×10^{-12} g, and the drop of histone-serum albumin (diameter 1.98 μ, weight 1.8×10^{-12} g).

A yeast cell of a thermophilic culture of Saccharomyces cerevisiae weighs 31.0×10^{-12} g, the cell diameter is 7.0-7.4 μ, and a drop of serum albumin-gum has a diameter of 6.82 μ and a weight of 33.7×10^{-12} g. These indices are also characteristic of an erythrocyte, whose diameter is 7 μ and weight 31.4×10^{-12} g [155, 157, 442].

Such values can also be found in many other drops of proteins-carbohydrates, proteins-proteins, etc.

5. It is interesting to note that the decrease in concentration with increasing size is observed not only in cells, as was shown by Mitchison et al. [38, 400], but also in such simple models as coacervate drops.

Morovitts and Turtelott [237] have noted that "as was stressed several years ago by the British mathematician biologist D'Arcy Thompson, the chief criterion in comparing the size of live objects is their mass, the latter being proportional to the cube of the length" (p. 106).

Unfortunately, this proportionality is not always observed [38, 129, 407].

We hope that if suitable mathematical formulas become available, it will be possible to calculate and predict the weight and concentration of the dry matter of cells and model systems as a function of the change of their volumes.

Unfortunately, the method of interference microscopy can be used to measure only the total concentration of all the substances. It isnot clear how individual chemical compounds, particularly nucleic acids, will behave in the drops.

Chapter 5

CONCENTRATION OF NUCLEIC ACIDS IN COACERVATES

Nucleic acids take a direct part in the synthesis of protein in the processes of the transmission of hereditary properties of organisms, and can also act as donors of mononucleotides, which most frequently function as coenzymes of various systems [29, 30, 132, 181, 313, 359-362, 408]. When the nucleic acids of phagi and viruses enter the organism, they become matrices on which the cell enzmyes build molecules similar to them [416]. Thus, together with protein, they play a leading role in biological processes. Like polypeptides, nucleic acids and polynucleotides can be synthesized abiogenically [162, 182, 276, 277, 348, 351, 631, 833, 860-862, 881, 885-888]. They form various complexes with acid and alkaline proteins, and readily give coacervates over a wide pH range (from 1.2 to 9 and higher) [901-902, 906].

The distribution of nucleic acids between the drops and the equilibrium liquid was studied in two-component protein-carbohydrate, protein-nucleic and multicomponent coacervates. The nucleic acids were determined in toto in drops and in the equilibrium liquid [129, 217, 258, 335].

Separation of the drops from the equilibrium liquid is achieved by contrifuging or by allowing the liquid to stand.

In this case, the drops deposit on the bottom and walls of the container. The quantity of nucleic acid is calculated from the nucleic phosphorus, which can be found by various methods.

The total volume of all the coacervate drops is much smaller than the volume of the equilibrium liquid surrounding them. Therefore, in order to obtain comparable data, the content of nucleic acids is recalculated per unit volume equal to 1 ml, for both the drops and the equilibrium liquid, and if necessary for the entire coacervate and mixture of solutions. /104

It has been shown that nucleic acids concentrate mainly in drops, and that the content of the same nucleic acid in these drops may change with the chemical composition of the coacervate and the conditions of its preparation [129].

Some results on the distribution of nucleic acids in coacervates are given in Table 20.

From the figures listed in Table 20 it follows that the RNA concentration of the drops increases tens of times as compared to the intial solutions from which they were formed.

Conversely, the RNA concentration in the equilibrium liquid is much lower than the solutions, and hundreds of times lower than that in the drops.

Such a distribution of nucleic acids is observed when they are added to the solutions up to the formation of coacervates.

The behavior of nucleic acids added to the finished coacervate system containing drops was studied by means of ordinary and radioactive RNA, tagged at the phosphorus [129, 211].

Table 20

Distribution of RNAin Coacervates*

Content, mg/ml		RNA, %/ml		C_{drops} / $C_{solution}$	C_{drops} / $C_{equilibrium\ liquid}$
drops	equilibrium liquid	drops	equilibrium liquid		
histone - RNA					
67.6	0.44	99.3	0.7	24	154
gelatin - gum					
2.44	0.009	99.6	0.4	41	271
gelatin- gum - β-amylase - starch					
2.09	0.0019	99.8	0.2	35	152

*C - concentration; $C_{solution}$ - initial RNA concentration of the mixture. Numerical data for the histone - RNA coacervate borrowed from Serebrovskaya [329].

Distribution and interaction of radioactive and ordinary ribonucleic acids in coacervates.* The preparation of radioactive RNA tagged at the phosphorus was isolated from yeast. The yeast was grown on amedium containing p^{32} [56]. The tagged nucleic acid, RNA-P^{32}, was added to a gelatin-gum coacervate and after an incubation of 15 min at 42°, the radioactivity was determined. The number of pulses obtained was recalculated for 1 mg of RNA. /106

In calculations of the RNA content per unit volume (1 ml), the following data were used: the volume of the entire coacervate was 5.2 ml, of the equilibrium liquid 5 ml, and of the fraction of drops, 0.2 ml (Table 21).

It follows from the data of Table 21 that radioactive RNA added to the entire coacervate penetrates the drops and concentrates in them. The greatest increase of RNA-P^{32} in drops was observed at its lowest content in the solution from which the coacervate was obtained. A further increase of RNA-P^{32} in the entire system leads to an increase of the absolute amount of RNA-P^{32} in the drops, but at the same time there takes place a decrease in the ratio of RNA-P^{32} of drops to RNA-P^{32} of the coacervate and equilibrium liquid, i.e., the degree to which RNA-P^{32} concentrates in drops decreases as a result of the high saturation of the entire system with RNA-P^{32}.

*The study of the behavior of nucleic acids added to the finished coacervate containing drops was carried in cooperation with V. Liebl in 1960 in the I. Khaloupka Laboratory at the Biology Institute of the Czechoslovak Academy of Sciences (Prague).

Table 21

DISTRIBUTION OF RNA-P^{32} IN COACERVATE (in mg %)

Equilibrium liquid	Drops	drops / solution	drops / equilibrium liquid
11.8	283	13.2	23.9
20.6	482	12.0	23.4
33.8	670	10.7	19.8
48.7	793.5	9.6	16.8

The ability of RNA to pass from the equilibrium liquid into the drops suggests the reverse process, i.e., the possibility of RNA passing from the drops into the equilibrium liquid.

The question of an interaction between nucleic acids within the same coacervate system thus arises.

Interaction of Nucleic Acids in Coacervates. The following experiments were set up to elucidate the possibility of interaction between RNA molecules in coacervates.

To a previously incubated coacervate system already containing radioactive RNA, ordinary RNA obtained from the same batch of yeast by the same method was added. The incubation was then repeated (Table 22).

It follows from the data of Table 22 that ordinary RNA additionally introduced into a coacervate already containing radioactive RNA disturbs the equilibrium and displaces RNA-P^{32} from the drops. The radioactivity in the drops decreases. A part of the radioactive RNA passes into the equilibrium liquid, and the radioactivity of the latter increases. The RNA molecules in the coacervate are in a dynamic equilibrium and can pass from the equilibrium liquid into the drop and vice versa. /106

These results pertain as a whole to all the drops, since chemical methods cannot be used to determine the amount and characteristics of distribution of nucleic acids in individual drops.

Concentration of Nucleic Acids in Individual Coacervate Drops. At the present time, there are many data on the content of nucleic acids in cells and subcellular structures in various organisms, but at the same time there is a lack of information on the amount and concentration of nucleic acids in individual coacervate drops. This last point is particularly important, since many biological systems which involve the participation of nucleic acids may also be built in accordance with the coacervate type [210, 923-936]. The study of nucleic acids in various coacervate drops was made by using the method of ultraviolet microscopy.

Table 22

INTERACTION OF NUCLEIC ACIDS IN COACERVATES (in pulses/min per 1 mg)

System	RNA-P^{32}	RNA-P^{32} + RNA
Coacervates	1978	1978
Drops	1040	551
Equilibrium liquid	935	1417

Brief Description of Ultraviolet Microscopy as a Method for Determining the Content of Nucleic Acids Down to 10^{-13} g in Coacervate Drops and Cells

The beginning of studies of biological structures of cells in ultraviolet light dates back to the 1930's-1940's.

In 1936, in Sweden, Caspersson and three years later Brumberg, who can rightfully be considered the founder of ultraviolet microscopy in our country, created the first models of the ultraviolet microscope [59-61, 619-621].

At the present time, ultraviolet microscopy is widely used in cytochemical studies of cells [63, 64, 66-72, 432, 496-497]. The method essentially consists in measuring the amount of ultraviolet light absorbed by the cells. /107

Hence, in any microscopic object, structures can be observed containing substances absorbing ultraviolet rays. Such substances also include purine and pyrimidine bases, which enter into the composition of nucleic acids [65, 117, 128, 405, 636, 961]. Purines and pyrimidines have a principal absorption maximum in the wavelength range of 248-280 mμ, and nucleic acids, at 260-265 mμ, and they have a very high absorption coefficient [620, 831]. Owing to such properties, as little as 10^{-13} g of nucleic acids can be determined by means of modern instruments. Wilkins contends that the absorption of structures a fraction of a micron in size, about 0.25 mμ, can be measured with ultraviolet microscopes. The study of such fine structures is of great interest, but it has not yet been sufficiently developed [118, 246].

The determination of RNA and DNA in individual cells by means of ultraviolet rays is very simple as compared to cumbersome biochemical analyses. In addition, biochemical methods cannot provide an answer to the question of the content of RNA and DNA in individual cells and require the consumption of a large amount of material, on the order of hundreds of billions of cells [26, 684, 734].

Using ultraviolet microscopy, it is necessary to consider the possibility of the presence in the cells of not only nucleic acids, but also other compounds absorbing ultraviolet rays [51, 54].

For this reason, it is frequently necessary to subject the histological preparations to a long treatment [76, 102, 297, 316]. At the same time, a quantitative determination of RNA and DNA by such chemical methods as those of Schmidt and Thannhauser, Ogur and Rosen and also by analyzing purines and pentoses frequently causes considerable distortions of the results as a result of the presence of various impurities in the preparation [245, 866]. The most accurate method is that of Elson and Chargaff. In this case, the RNA and DNA are found by summing up the constituent mononucleotides. However, this method is very cumbersome [133]. /108

Tables 23 and 24 present comparative data on the determination of the content of nucleic acids by various methods [246, 955].

Table 23

CONTENT OF NUCLEIC ACIDS IN RATE LIVER CELLS (in g/100 g of raw tissue)

Comparsion of Methods		Comparison of Methods	
ultraviolet microscopy	chemical analysis	ultraviolet microscopy	chemical analysis
1.37—1.58	1.42—1.67	1.45—1.44	1.20—1.41
1.28—1.50	1.38—1.63	1.41—1.66	1.43—1.63
1.16—1.46	1.31—1.55	1.49—1.66	1.47—1 73

The data of Tables 23 and 24 indicate that discrepancies in the individual determinations of nucleic acids within each method are approximately of the same order as between results obtained by chemical analyses and by ultraviolet microscopy. For ultraviolet microscopy, the errors of the extreme values range from

Table 24

CONTENT OF NUCLEIC ACIDS IN 10^{-12} g PER CELL

Specimen	Comparison of methods	
	ultraviolet microscopy	chemical analysis
	DNA	DNA
Bull liver	5.9∓0.11	6.4
Rat liver	6.0∓0.4	8.6
Bull thymus gland	5.7∓0.22	6.4
Bull sperm	3.4∓0.4	3.3
Sheep sperm	2.9∓0.3	5.4
Rat sperm	3.1∓0.5	7.2—6.6
Frog erythrocytes (Rana temporaria)	8.2∓0.68	8—9
Chick erythrocytes	3.1∓0.5	2.4
Triton erythrocytes	45.0∓0.5	48
	DNA	RNA DNA RNA
Ehrlich ascites cancer	14.0∓1.5	4.0∓0.4 12.9 4.4
BA-ascites tumor	6.8∓0.6	2.0∓0.2 6.6 1.6

1 to 10%, and the average errors are 5% of the value being determined. Thus, both ultraviolet microscopy and chemical methods have their advantages and disadvantages. The two methods can be used equally sell for the determination of the average RNA and DNA content in cells.

Table 25 gives only some necessary data on the quantity of nucleic acids in biological specimens.

The data of Table 25 indicate that the content of nucleic acids in cells and subcellular structures varies over a wide range.

At the same time, it is interesting to have an idea of the changes which can be observed in the content of nucleic acids as a function of the size of the cell in the same tissue or in the same microorganism. Data on this problem are given in Table 26 [959].

Thus, as the volume of the cells increases, the content of nucleic acids increases; at the same time, their concentration, calculated per unit volume, decreases [948]. /109

It is well-known that the amount of DNA in a cell is determined by the number of chromosomes. In diploid ones, the quantity of DNA is twice the number in haploid ones. If the cells have the same number of chromosomes, for example, a haploid number, then independently of the size of the nucleus, the DNA content will be the same [831]. Hence, as the size of the nuclei increases, the DNA concentration calculated per unit volume will decrease [770, 771, 863, 878, 907]. /113

Frequently, the cell volumes cannot be calculated, and therefore the RNA and DNA content per unit area of the cell is compared. These relationships are not always observed [53, 54]. Figure 41 shows results of a study by Hyden [727] on hypoglossal cells, which confirm the relationship described earlier. Unfortunately, such data are scarce in the literature, since the majority of researchers give results recalculated

Table 25

CONTENT OF NUCLEIC ACIDS IN CELLS AND SUBCELLULAR STRUCTURES

No.	Specimen	Nucleic acid		Reference
	Bacteria, sizes 2-20 μ, average 10-24% [459]			
		RNA + DNA		
1	B. acethylicum, B. megatherium	10.2-10.3%		[28]
2	Sarcina luteae, B. mycoides	11.4-11.9%		[28]
3	Myxobacterium sorangium	12.6%		[28]
4	Proteus vulgaris	13.0%		[28]
5	Micrococcus candicans, B. fluorescens	14.0-14.2%		[28]
6	B. pyocyaneum, Spirillum volutans	21.6-28.8%		[28]
		RNA	DNA	
7	B. tuberculosis	0.9%	1.4%	[624]
8	E. Coli, staphylococci and typhus bacilli	5-10%	3-4%	[946]
9	Schigella paradysenteria	4.09%	0.74%	[183]
10	B. typhi (different strains)	8.8%	4.03%	[184]
11	Azotobacter (Azotobacter chroococcum, agile, vinelandi	$8.4\text{-}5.5 \cdot 10^{-14}$ g per cells 3.6-6.36%	$3.4\text{-}4.2 \cdot 10^{-14}$ g per cells 0.81-1.7%	[31]
		RNA + DNA		
12	Thermophiles corresponding to cellulose bacteria	6%		[258]
		RNA	DNA	
13	B. licheniformus F	2.23%	0.21%	[137]
	thermophiles	$2.81 \cdot 10^{-14}$ g per cells	$0.26 \cdot 10^{-14}$ per cells	
	mesophiles	5.58%	0.56%	
		$10.2 \cdot 10^{-14}$ g per cells	$1.0 \cdot 10^{-14}$ g per cells	
	Fungi, sizes 7-14 μ and higher			
		RNA		[144]
14	Yeast (Saccharomyces cerevisiae) thermophiles	3.53%		
		$1.09 \cdot 10^{-12}$ g per cells		[215]
	mesophiles	4.45%		
		$1.82 \cdot 10^{-12}$ g per cells		
	Mold fungi			
		RNA + DNA		
15	Aspergillus oryzae	3-6%		[28]
16	Penicillum glaucum	3-6%		[459]
		RNA	DNA	
17	Aspergillus fumigatus			
	thermophiles	1.8%	0.24%	[145]
	mesophiles	2.6%	0.80%	[145]

Table 25 (cont.)

No.	Specimen	Nucleic acid		Reference
		RNA + DNA		
18	Spores Fuligo varians	7.9%		[28]
19	Plasmodium reticularia lycoperdon	3.68%		[28]
	Algae, size 5 μ and higher			
		RNA	DNA	
20	Blue-green Aphanizomenon flosaque	3.4%	1.19%	[338]
21	Mastigocladus laminosus Cohn			
	thermophiles	0.23%	0.05%	[137]
	mesophiles	0.27	0.09	
22	Diatoms	0.21-1.44%	0.84-3.96%	[337]
23	Green Scenedesmus quadricanda	3.20%	1.30%	[337]
24	Chlorella	2.81%	0.53%	[291]
25	Brown Cystosira barbata	0.21%	0.53%	[337]
	Higher plants			
		RNA	DNA	
26	Cells of root around rootcap	14.2-18.0%	3.5-4.7%	
		$0.84 \cdot 10^{-12}$ g per cells	$3.44 \cdot 10^{-12}$ g per cells	[179]
27	Inflorescences of Salix caprea	0.91%	2.18%	[337]
28	Inflorescences of Almus barbata	1.67-0.89%	2.43-2.08%	[337]
		RNA + DNA		
29	Poppy ovules	4.87%		[28]
		RNA + DNA		
30	Pine kernel germ	6.8%		[28]
		RNA	DNA	
31	Wheat germ	2.89%	2.5%	[27]
32	Pea sprouts	1.58-4.2%	--	[179]
33	Potato sprouts	0.725%	0.048%	[179]
34	Pollen of dicotyledons	0.6-1%	--	[77]
		. . . . .		[765]
		. . . . .		[825]
	Plastids, size 1-24 μ			
		RNA		
35	Tobacco chloroplasts	3.0-4.0%		[350]
36	Sunflower	1.42-1.54%		[349]
37	Pea	2.0-4.0%		[352]
38	Lucerne, spinach, sugar beet	0.96-1.00%		[81]
39	Carrot chromoplasts	0.72-1.34%		[918]
40	Pepper chromoplasts	1.24%		[81]
41	Leucoplasts of sugar beet petiole	0.99%		[797]

Table 25 (cont.)

No.	Specimen	Nucleic acid	Reference
		Animal cells	
42	Cytoplasm of mouse liver cells	RNA + DNA $1.7 \cdot 10^{-14}$ g/μ^3	[246]
		Nuclei, size 1–100 μ	
		DNA	
	Erythrocytes	$1.97–15.0 \cdot 10^{-12}$ g same as above	[945]
43	Carp erythrocytes	$3.20 \cdot 10^{-12}$ g " " "	[945]
44	Pike erythrocytes	$1.70 \cdot 10^{-12}$ g " " "	[945]
45	Trout erythrocytes	$4.9 \cdot 10^{-12}$ g " " "	[945]
46	Chick erythrocytes	$3.1 \cdot 10^{-12}$ g " " "	[100]
47	Leucocytes	$6.64–7.30 \cdot 10^{-12}$ g " " "	[431]
48	Spermatozoa	$0.33–3.42 \cdot 10^{-12}$ g " " "	[431]
49	Carp spermatozoa	$1.6 \cdot 10^{-12}$ g " " "	[945]
50	Pike spermatozoa	$0.85 \cdot 10^{-12}$ g " " "	[945]
51	Trout spermatozoa	$2.45 \cdot 10^{-12}$ g " " "	[945]
52	Calf thymus gland	$1.1 \cdot 10^{-12}$ g " " "	[864]
53	Pancreas	$2.7–7.38 \cdot 10^{-12}$ g " " "	[431]
54	Spleen	$2.6–6.55 \cdot 10^{-12}$ g " " "	[431]
55	Heart	$2.7–7.38 \cdot 10^{-12}$ g " " "	[431]
56	Mouse liver	$3.64 \cdot 10^{-12}$ g " " "	[863]
57	Rat liver	$7.8–6.6 \cdot 10^{-12}$ g " " "	[51, 58]
58	Mouse skin	$6 \cdot 10^{-12}$ g " " "	[246]
59	Nerve cells	$45–1500 \cdot 10^{-12}$ g " " "	[396]
		RNA DNA	
60	Gangliar cells of retina	$2.57 \cdot 10^{-12}$ g $5.8 \cdot 10^{-12}$ g	[53]
		RNA	
61	Motoneurons, size 20–100 μ	0.5–1.1%	[52, 58]
62	Ehrlich acytes tumor (mouse skin)	$13 \cdot 10^{-12}$ g	
63	Mouse sarcoma	$18 \cdot 10^{-12}$ g	[246]
		Nucleoli, size 0.6–1.45 μ	
		RNA	
64	Gangliar cells of retina	$2.5–7 \cdot 10^{-12}$ g	[53]
		Mitochondria, size 0.2–5 μ	[179, 463 635 765 801]
		RNA	
65	Higher plants	1%	[352]
66	Animals	0.5%	[212]

Table 25 (cont.)

No.	Specimen	Nucleic acid	Reference
	Mitochondria, size 0.2-5 μ		[246, 909]
		RNA	
67	Bacteria	30-70%	[352]
68	Yeast	42%	[352]
69			
70	Wheat, clover, pea	30-45%	[352]
71	Spinach	42-45%	[782]
72	Animals	30%	[100]
73	Rat liver	45-50%	[352]
		RNA	
74	Viruses	1-30%	[416, 892]
		DNA	
75	Phages	9-40%	
76	Mycoplasm, size 0.25 μ M. Gallisepticm	RNA 4% DNA 8%	[373] [237, 875]

Table 26

CONTENT OF NUCLEIC ACIDS
AS A FUNCTION OF THE SIZE OF THE SPECIMEN

Specimen	Size	Amount, %		Reference
		RNA	DNA	
Yeast, Saccharomyces cerevisiae Strain 239	Volume, 10^{-12} cm³	11.4	0.86	[959]
	5—45	(0.57)	(0,043)	
	65—145	1.03	0.030	
		(1.5)	0,049)	
Nuclei of motoneurons	Area, 10^{-8} cm²			
	110	--	1.1	[58]
	160	—	0.75	
	220	—	0.5	

*Figures in parentheses-content of nucleic acid 1.10^{-12} g

for nucleic acids in "arbitrary units" [831] or 10^{-6} g of phosphorus. Although the nature of the relationships remains the same, the material cited is less convincing [245].

Thus, by combining chemical methods and ultraviolet microscopy, one can form an idea of the quantity of nucleic acids in individual cells and subcellular fractions. /114

All these data are cited in order to be able to compare them with results obtained on model coacervate systems whose composition includes nucleic acids.

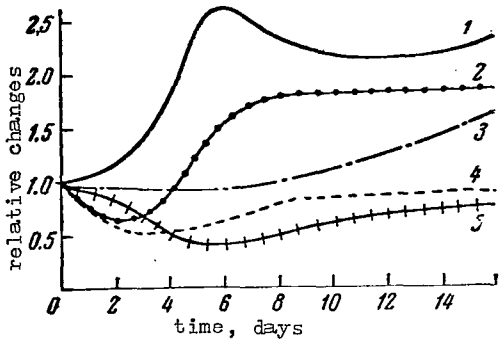


Figure 41. Diagram of the Distribution of Changes in Hypoglossal cells 1-cell volume; 2-protein content of cell; 3-RNA content of cell; 4-concentration of dry mass; 5-RNA concentration

Method of Determining Nucleic Acids in Individual Coacervate Drops. The determination of nucleic acids in coacervate drops was carried out in ultraviolet microscopes and cytospectrophotometers by a qualitative and quantitative method.

Five different models — two mass produced ones MUF-2 and MUF-4, and three experimental ones — were employed for this purpose. Thanks to the use of different designs, measurements of nucleic acids in coacervate drops from 1 to 200 mμ in diameter were successfully carried out [2, 6, 24].* Comparatively large sizes of coacervate drops make it possible to study them under MUF-2 and MUF-4 ultraviolet microscopes. In these models, the source of ultraviolet light are high pressure mercury-quartz lamps. Usually, PRK-4 and SVD-120, SVD-120A lamps are employed [1, 88]. Each of them has its advantages and disadvantages. It should be pointed out that the SVA-120A lamp has a higher intensity of ultraviolet light than PRK-4 or SVD-120. In visual observations, the visible rays are separated with a neutral light filter and the ultraviolet ones with a UFS-1 light filter [71] and a quartz cell filled with chlorine. The appearance of coacervate drops containing nucleic acid upon their illumination with ultraviolet light is shown in Fig. 28 c. In this case, the ultraviolet light which has traversed the field of view enters a luminescent converter whose composition includes certain luminors [200, 302], i.e., compounds which are excited by ultraviolet light and emit radiation in the visible range.

The yield of visible light as compared to the intensity of the incident ultraviolet light is very low, but sufficient for observations. As shown in Fig. 28 c, droplets with nucleic acids are colored red. If nucleic acid is present not only in the drops but also in the equilibrium liquid, the field of view is also colored pink or red.

The color intensity of the drops and field depends on the amount of nucleic acid. For one of such coacervates, consisting of protein, carbohydrate and RNA, it is shown /115 that in the presence of 48.75 mg % of nucleic acid, the latter passes completely into coacervate drops. When the nucleic acid concentration is raised to 192.5 mg %, part of its remains in the equilibrium liquid [138]. In addition, black-and-white pictures of specimens photographed at various wavelengths can be obtained on MUN-2. Figure 42 shows black-and-white photographs of drops with nucleic acids. Using a chromoscope attached to the MUN-2, from the black-and-white positive one can obtain

*The measurements were made primarily in the ultraviolet microscopy laboratory of Ye. M. Brumberg at the State Optical Institute im. S.I. Vavilov in Leningrad in cooperation with L.S. Agronskiy, M.P. Bukhman, I.L. Zarubina and N.V. Korolev and at the Animal Biochemistry Departments of Leningrad University and the Chemistry Department of Moscow University, and also in a plant laboratory.

a color picture of drops corresponding to their appearance under the ultraviolet microscope with the luminescent converter. In the chromoscope, the black-and-white spots on the plates are transformed by means of colored filters and give a color image of the object [24, 69]. The use of the chromoscope is due to the fact that the direct photographing of the color image of the object (drops) in the field of view of the microscope requires a long exposure, so that an undersirable secondary effect of ultraviolet light is manifested which frequently causes the cells, nuclei, drops and even chemical compounds to break down [17, 64, 75, 76, 205, 227]. A quantum of light with λ = 250 mμ is capable of breaking 20 hydrogen bonds in polymeric nucleic acid [20]. Prolonged irradiation is associated with the breadkdown of amino acids; for example, sulfur is evolved from cysteine. In order to shorten the irradiation time, the focusing is performed in visible light [71, 72].

The photographs shown in Figs. 28c and 42 show the appearance of coacervate drops containing not only nucleic acids, purines, pyrimidines and nucleotides, but also any compounds absorbing ultraviolet light.

Quantitative Content of Nucleic Acids in Drops. The amount and concentration of nucleic acids in unstructured globular drops can be determined from the transparency and optical density [121, 129, 138]. The concentration was calculated from the Lambert-Bouger-Beer law [94, 405, 470]:

$$I = I_0 \cdot 10^{-(x_1C_1+x_2C_2+\ldots+x_nC_n)\cdot d}, \quad (1)$$

where I_0 is the intensity of incident light; I is the intensity of light after it has traversed a layer of thickness d; C is the concentration of the substance; X is the extinction (absorption) coefficient for measuring a given wavelength.

The degree of transparency or optical density can be measured from spectra or directly.

The nucleic acids were determined from spectra using an ultraviolet microscope with a spectral quartz attachment designed by Brumberg and Gershgorin [65, 71]. /116

To this end, the following items were prepared: 1) coacervate from 0.67% solutions of gelatin and gum (in the proportion of 5:3) and RNA at pH 3.5-4.0 and 42°. The RNA concentration was 48.75 mg %; 2) the same coacervate without RNA (blank); 3) RNA solution with a known concentration of nucleic acid, used for calculating the extinction coefficient, indicated in formula (1).

Figure 43 shows photographs of drop and its spectrum. Droplets consisting of gelatin-gum practically do not absorb ultraviolet light in the 280-250 mμ range; on the other hand, drops with nucleic acid and the RNA solution display a strong absorption in this region.

Thus, the blackening of spectral lines (see Fig. 43) results from the absorption of ultraviolet light by nucleic acid present in the drop.

The degree of blackening was measured photometrically with a microphotometer. The content and concentration of RNA in the drop were calculated from the data obtained.

Shown below are the results of photometric analysis of the spectra and the procedure used for the calculations (see formulas 2-3) for a drop 29 mμ in diameter [138].

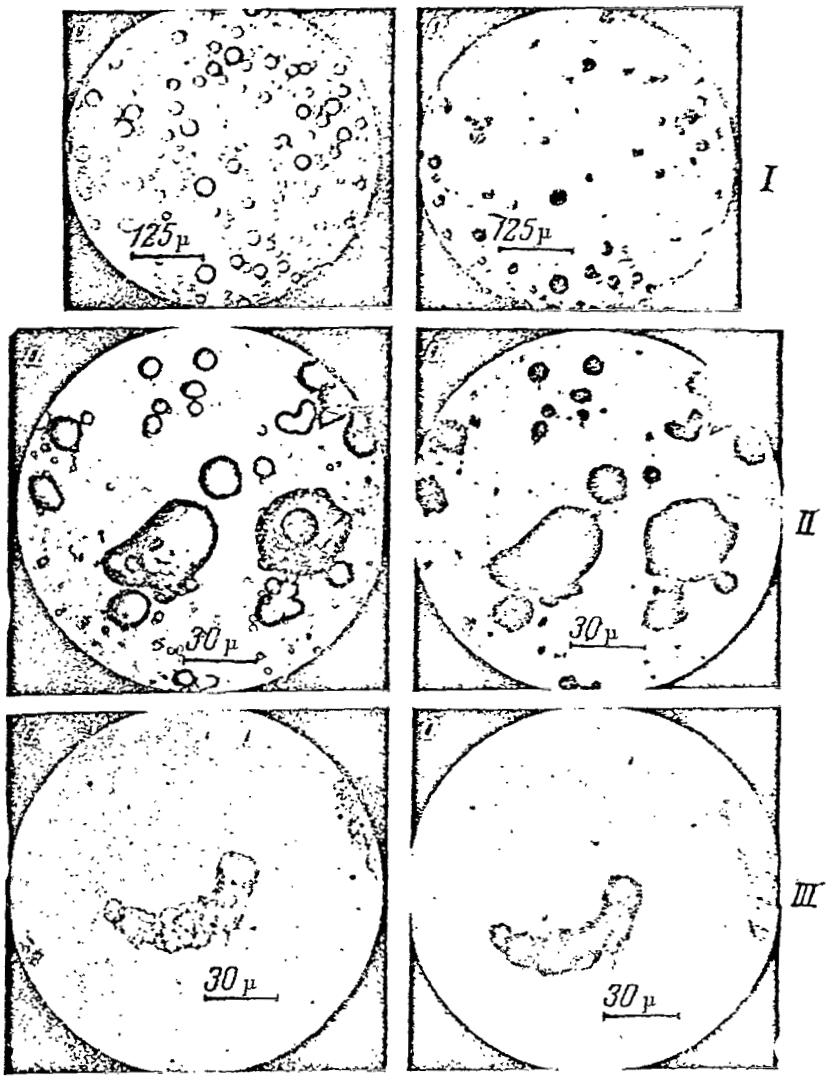


Figure 42. Coacervate Drops with Nucleic Acid
I) RNA-gelatin-gum; II) RNA-gelatin-gum; III) DNA-histone;
a-in visible light; b-in ultraviolet light

Object	$\log I/I_0 \cdot 100$ at $\lambda = 280$ μ
Nucleic acid solution	1.00
Drop containing nucleic acid	1.39
Drop without nucleic acid	1.94

The extinction coefficient (x) can be expressed as follows:

$$x = \frac{2-(\lg I/I_0 \cdot 100)}{C.d} = \frac{2-1.0}{390 \cdot 0.01} = 0.25 \text{ cm/mg \%}, \quad (2)$$ /118

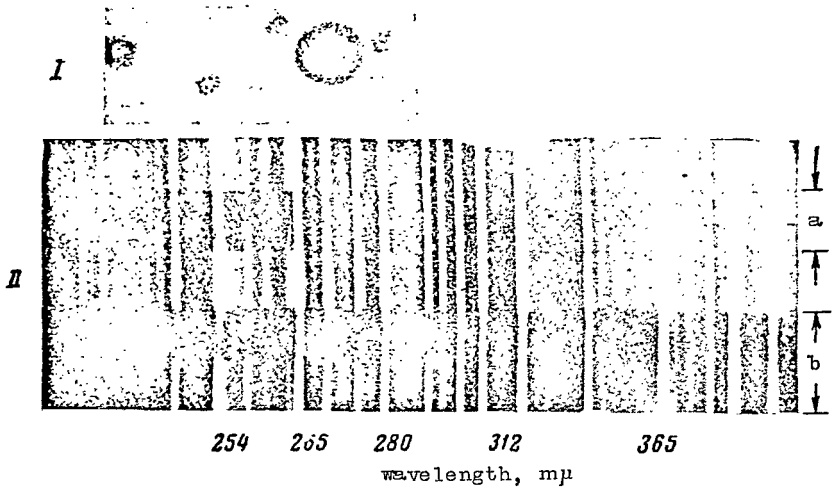


Figure 43. Absorption of Ultraviolet Light by Coacervate Drops
I-drop with nucleic acid; II-spectrum of drop with nucleic acid;
a-drop; b-gradual attenuator

where I/I_0 is the attenuation of light by the RNA solution; C is the concentration of the RNA solution = 390 mg %; d is the thickness of the solution layer, 0.01 cm.

After some simple transformations of formula (1), the RNA concentration in the drop is calculated from the formula

$$C_1 = \frac{\lg(I_2/I_0 \cdot 100) - \lg(I_1/I_0 \cdot 100)}{-x \cdot d} = \frac{1.94 - 1.30}{0.25 \cdot 29 \cdot 10^{-4}} = 760 \text{ mg \%}, \quad (3)$$

where: I_2/I_0 is the attenuation of light by the drop without nucleic acid; I_1/I_0 is the attenuation of light by the drop with nucleic acid; d is the drop diameter = 29 mμ.

Thus, the RNA concentration in the drop increased by a factor of 15 as compared to the initial solutions from which it was obtained.

Formula (3) does not take into consideration the change in the concentration of gelatin and gum arabic upon addition of RNA, or the attenuation of light due to reflection on the surfaces of the drops.

However, this cannot introduce any approciable error, since the extinction coefficient of gelatin and gum arabic is too small at 280 mμ, and the reflection of light on the surfaces is so slight that the error due to this factor will be considerably less than many other errors arising during the photographing of the spectra and their photometric analysis [52, 57, 58].

The total error for coacervate drops was 5% of the value of the attenuation of light being determined.

However, although this method is simple from the standpoint of the design of the instrument, it is quite labor-consuming and does not permit one to take a large number of measurements of drops in a short time.

The principal data on the concentration of nucleic acids in the drops were obtained by direct measurement of the optical density in cytospectrophotometers.

Determination of Nucleic Acids in Coacervates by the Cytospectrophotometric Method. Cytospectrophotometers are photoelectric instruments consisting of a microscope and spectrophotometer. They are essentially microspectrophotometers permitting the determination of substances in cells from the absorption of light with a great accuracy [2, 3]. The nucleic acid content of coacervate drops was determined from the optical density, measured from spectra, and by direct reading of probe cytospectrophotometers designed by Korolev and Agroskin [2, 3, 6, 185, 186]. In order to determine the optimum conditions of measurement of the optical density in these instruments, photographs of absorption spectra of nucleic acids, purines and pyrimidine bases and mononucleotides were taken in both the drops and the equilibrium liquid and solutions. The absorption spectra were studied within a cytospectrophotometer with an oscillograph. The light source used was a hydrogen lamp giving a continuous radiation spectrum in the 240-310 mμ range. Adenine, hypoxanthine, guanine, cytosine, uracil, thymine and also adenosine and the mononucleotides adenylic, cytidylic, guanylic and thymidylic acids, RNA and DNA [42, 129, 626], were studied in a coacervate from gelatin and gum arabic. To this end, the mononucleotides were dissolved in 0.67% solutions of gum, RNA and DNA in a 0.1 M solution of sodium acetate, and the purine and pyrimidine bases, in 0.1 N HCl. A 0.67% solution of gelatin /119 was then added, and drops were formed at pH 3.8-4.1 and 40-43°. Several coacervates with different initial concentrations of each compound not above 0.08% in the entire coacervate were prepared. The lowest solubility is displayed by guanine, but the guanine concentration employed was completely measurable in the cytospectrophotometer. Figure 44-46 show photographs of an oscillogram of the coacervates and also absorption curves obtained after processing the oscillograms. It was found that guanine, cytosine, uracil, adenosine, adenylic, cytidylic and guanylic acids are more or less uniformly distributed between the drops and the equilibrium liquid, since the absorption curves on the oscillograms for the drops and the equilibrium liquid are nearly the same. At the same time, adenine, hypoxanthine and thymine were found primarily in the drops, although they were present in significant amounts in the equilibrium liquid. RNA and DNA were chiefly concentrated in the drops, and coacervates can be obtained in which RNA and DNA were observed only in the coacervate drops and were practically absent from the equilibrium liquid.

Spectra of 0.1% solutions of DNA and RNA in acetate buffer were similar to the spectra of the coacervate drops. No significant shifts were observed in the absorption peaks of RNA and DNA in the coacervate drops. For this reason, the RNA and DNA contents in coacervate drops from various coacervates were determined by measuring the optical density at 265 mμ. In this case, the light source used was an SVD-120A mercury-quartz lamp, which made it possible considerably to increase the accuracy of the measurement of optical density by decreasing the size of the light probe. In probe spectrophotometers, the rays of light are directed through the object in the form of a light beam, a probe of definite diameter. The light probe is focused in the form of a small area at the center of the coacervate drops, and it radiates light beams along the diameter of the drop. If the light probe is passed through a drop, part of the rays are absorbed by the drop, and the light flux (I_1) emerging from the drop is attenuated. At the same time, the

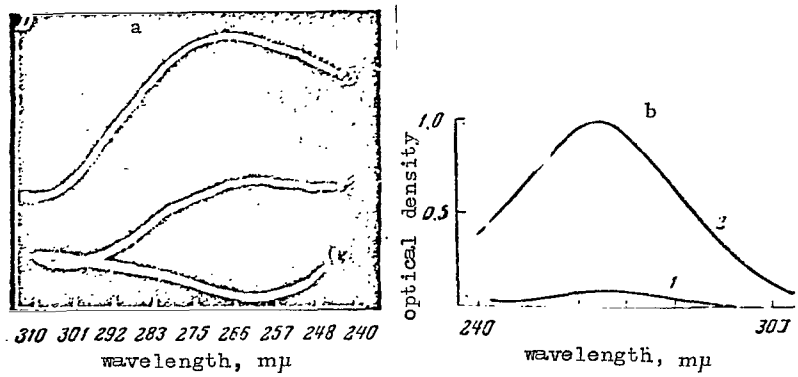


Figure 44. Coacervate Drops with RNA
a-oscillogram; b-absorption curve
1-equilibrium liquid; 2-3-drops

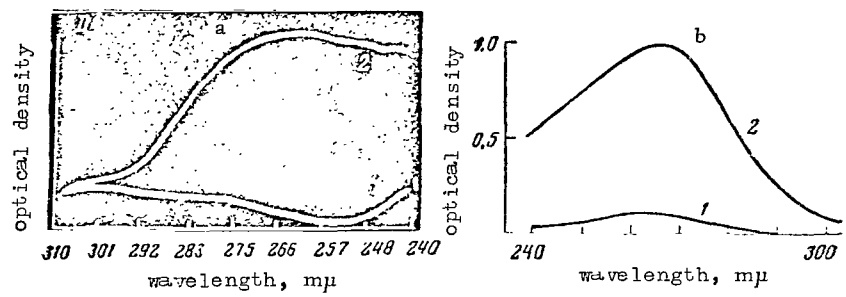


Figure 45. Coacervate Drops with DNA
a-oscillogram; b-absorption curve
1-equilibrium liquid, 2-drops

light flux (I_0) which has passed through the medium surrounding the drop is measured, and the optical density D is calculated from the formula $D = \log I_0/I_1$ (4). The error is no more than 2% of the value of the optical density being determined [4].

Thus, the measurement of the content of substances in the drop is made after the rays have travelled along its diameter. The narrower the area — the probe and cone, the more accurate the measurements. Indications by certain authors [5] that when a probe is too small as compared to the object there can be a marked light scattering were checked and not confirmed. If the probe is larger in size than the drop, part of the beams of the probe will pass through the medium surrounding the drop and thus distort the results. In this case, the so-called two-wave method is usually employed [4, 86], in which the measurements of the optical density of the object and medium are made at two different wavelengths in order to take the absorption of the medium into account. /120

In our study, use was made of probes having a diameter smaller than that of the drop. For large drops, a probe with a 10 mμ diameter was used, and for smaller

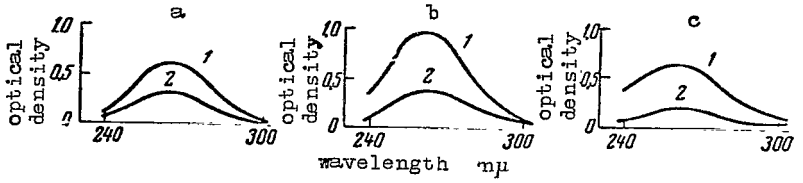


Figure 46. Absorption Curves for Adenine (a), Hypoxanthine (b) and Thymine (c)
1-drop; 2-equilibrium liquid

ones, 5mμ, 1 mμ and quartz objectives ×10 × 0.2, ×40 × 0.65 and ×65 × 0.8 were employed.

The concentration and RNA and DNA contents in individual drops were calculated from the formulas

$$C = \frac{D}{x \cdot d}, \tag{5}$$

$$P = \frac{C}{100} \cdot 4/3\pi r^3, \tag{6}$$

where D is the optical density at λ = 265 mμ; C is the concentration in %; d is the diameter of the drop in 10^{-4} cm; X is the absorption coefficient in %/g per cm; P is the weight in g; $4/3\pi r^3$ is the volume of the drop.

The absorption coefficients for preparations of nucleic acids were determined from solutions with a known concentration in both the SF-4 spectrophotometer and the cytospectrophotometer. For RNA, the absorption coefficient was 25,000 g/cm³, taken as 0.25 mg% for the calculations, and for DNA, 14,800, or 0.148 mg%, i.e., these values were close to the data available in the literature. It is shown that the absorption coefficients depend on the purity of the nucleic acid preparation, the degree of polymerization, the pH of the solution, and the presence of salts in the latter [129, 625, 831]. On the average, they range from 15,000 to 30,000 [101, 456, 954, 955].

The optical densities were measured in the range of 0.1 to 2.0. However, the most exact readings were those made at an optical density of 0.3 to 1.2.

The larger the absolute quantity of nucleic acids in the drops, the stronger they absorb ultraviolet light, and the optical density of the drop increases correspondingly.

For example, in a histone — RNA coacervate at an initial RNA concentration in the original solutions of 0.143%, a drop $14 \cdot 10^{-4}$ cm in diameter had a nucleic acid concentration of 5.7%, and the optical density was 1.98. Hence, in such a coacervate RNA should not be measured in drops having a diameter of more than 14 mμ, since these measurements exceed the range of the instruments. /122

For this reason, systems were prepared in which drops of different sizes in the same coacervate had optical densities in the indicated range.

In our study, the error of the optical density reading was 1-3% of the value being

determined. The total error due to measurement ot the diameter and optical density did not exceed 5% of the quantity of nucleic acids found.

Content and Distribution of Nucleic Acids in Drops

The content and concentration of nucleic acids in unstructured drops were studied in the following coacervates: histone — DNA, histone — RNA, clupein — DNA, clupein — RNA, gelatin — gum — DNA, gelatine — gum — RNA. The conditions of preparation and composition of the coacervates were the same as in the study of the total content of substances by interference microscopy.

The coacervates were prepared so that it would be possible to explain the capacity of each individual nucleic acid (RNA and DNA) to concentrate in drops with different proteins. Some of the results are summarized in Table 19 and Fig. 47-50. Exactly as in the case of determination of the dry weight of drops, the dotted lines on the graphs designate curves indicating the change in the content of nucleic acids in drops of various sizes, provided that the concentration of nucleic acid remains constant in all the drops. The concentration measured in the smallest droplet was taken as the constant value.

The solid line indicates the content of nucleic acids found in the determination of the concentration in each drop. Figure 49-50 show the dependence between the size of the drops and their RNA content in the same coacervate system.

In the same coacervate, the nucleic acid content in small drops is closer to the calculated data than in large ones.

This is due to the fact that the increase in the size of the drops proceeds not only at the expense of nucleic acid but also other compounds and water. Therefore, the concentration of nucleic acids in large drops is considerably lower than in small ones.

A similar conclusion was found to be correct in the case of different coacervate systems, for example, for histone — RNA and histone — DNA (Fig. 47-48).

The indicated dependence is also confirmed by the data shown in Tables 27 and 28. /123

Results of measurements of nucleic acids in coacervate drops showed the following:

1. In globular coacervate drops with diameters from 1 to 161 mμ, from $0.01 \cdot 10^{-11}$g to $708.6 \cdot 10^{-11}$ g of nucleic acids were found, and their concentration in the drops ranged from 0.24% to 31.3%. The amount and concentration of nucleic acids depend on the the size and chemical composition of the drops, and also on the initial concentration of RNA /124 and DNA in the solutions from which the drops are formed.

For example, in the clupein — DNA coacervate at a total DNA content of 0.83%, in a drop 4 mμ in diameter, the nucleic acid concentration was 8.3%. An increase in the DNA content to 0.143% in the solutions led to a twofold increase in the concentration in the drop. Whereas the intial solution contained 0.25% DNA, 31.3% DNA was observed in a drop 3 mμ in diameter. In the presence of a considerable excess, DNA and RNA appear in the equilibrium liquid. In most cases, the droplets in coacervates from histone, clupein, RNA and DNA are fine, and therefore the absolute amounts of nucleic acids present in them are small.

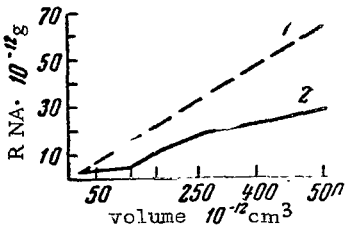


Figure 47. Coacervate Drops of Histone — RNA 1-content of nucleic acid in drops, calculated at a constant concentration measured in the drop of smallest diameter; 2-content of nucleic acid in drops, obtained experimentally

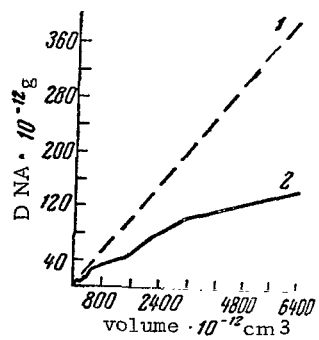


Figure 48. Coacervate Drops of Histone — DNA Notation same as in Fig. 47

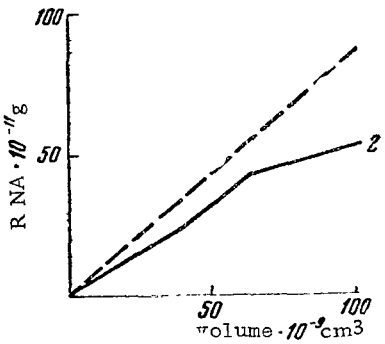


Figure 49. Coacervate Drops of Gelatin - Gum - RNA Notation same as in Fig. 47.

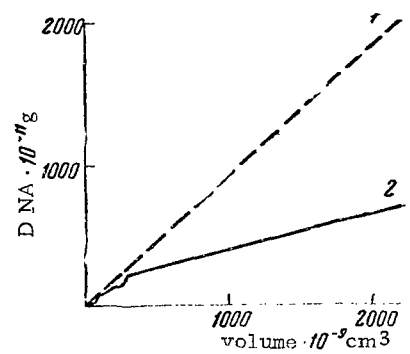


Figure 50. Coacervate Drops of Gelatin - Gum - RNA Notation same as in Fig. 47

The content of nucleic acids is much greater in large drops of gelatin — gum, and the concentration is considerably lower than in drops from alkaline proteins + RNA or DNA.

2. In all cases, coacervation is associated with a considerabley concentration of nucleic acids in the droplets (amounting to tens and hundreds of times the amount present in the initial solutions from which the drops were formed).

3. As the size of the drops increases, their concentration of nucleic acids rises, and the concentration per unit volume decreases.

4. Among the multitude of drops, there are some in which the concentration and RNA and DNA content are the same as in the cells and nuclei of many organisms.

For example, the nucleic acid concentration in bacteria 2 to 20 mμ in diameter ranges from 2 to 21.6%. The concentrations of nucleic acids in coacervate drops of /126

Table 27

NUCLEIC ACIDS IN UNSTRUCTURED COACERVATE DROPS

No.	Diameter 10^{-4} cm	Volume 10^{-11} cm^3	Optical density	Weight 10^{-11} g	Concentration %	$\frac{(C)\text{drops}}{(C)\text{solutions}}$
			Histone - RNA			
1	6	113.1	1.00	12.7	11.2	73
2	7	179.5	0.86	16.7	9.3	65
3	8	268	0.70	15.8	5.9	42
4	9	381	0.74	21.3	5.6	39
						$(C)_p^* = 0.143\%$
			Histone- DNA			
5	3	14.1	0.92	1.7	12.2	86
6	5	65.4	0.73	3.8	5.8	41
7	7	179.5	1.07	11.0	6.1	43
8	10	523.4	1.34	28.2	5.4	38
						$(C)_p = 0.143\%$
			Clupein - DNA			
9	2	4.2	0.37	0.5	12.6	152
10	4	33.50	0.49	2.8	8.3	100
11	5	65.4	0.49	2.4	6.7	81
12	8	268	0.68	15.5	5.8	70
						$(C)_p = 0,089\%$
			Clupein - RNA			
13	1	0.5	0.41	0.1	16.6	131
14	2	4.2	0.45	0.4	9.0	108
15	5	65.4	1.17	6.2	9.4	113
16	6	113.1	1.18	8.9	7.8	94
			Gelatin - gum - DNA			
17	15.8	2 100	0.1	0.9	0.43	24
18	35.6	23 700	0.17	7.6	0.32	18
19	55.4	89 700	0.29	31.7	0.35	19
20	59.4	109 800	0.24	30.0	0.27	15
						$(C)_p = 0,018\%$
			Gelatin - gum - RNA			
21	37.6	27 900	0.43	12.0	0.43	21
22	38.3	29 400	0.29	8.8	0.30	15
23	42.9	41 400	0.40	15.3	0.37	18
24	56.1	97 000	0.49	30.1	0.31	15
						$(C)_p = 0,02\%$

*$(C)_p$ concentration of nucleic acid of solution from which the drops were obtained.

histone — RNA, histone — DNA, clupein — RNA and clupein — DNA are very similar.

The diameters of such drops amounted to 2-16 mμ. From 1.5 to 31% of nucleic acids were found in the drops.

Frequently, as the volume of the cells and nuclei increases, their concentration of nucleic acids decreases.

Table 28

CONCENTRATION OF NUCLEIC ACIDS IN DROPS AS A FUNCTION OF THE RNA AND DNA CONTENT IN THE INITIAL SOLUTIONS

Composition	Diameter of drop, μ	Concentration, %	
		drop	solution
Clupein - DNA	4	8.3	0.083
		12.1	0.25
Histone - DNA	7	1.1	0.089
		9.3	0.143
Histone - RNA	8	2.5	0.083
		6.7	0.143
Gelatin - gum - DNA	15,8	0.43	0.018
		1.13	0.06

Thus, the character of the changes in the content and concentration of RNA and DNA in coacervate drops, depending on their size, is similar to the changes found for the dry weight and concentration of all the substances in the drops.

If the results of the determinations of the dry matter and nucleic acids in drops consisting of proteins and nucleic acids are compared, the protein content of these drops can be calculated.

Concentration of Protein in Coacervate Drops. The existing cytospectrophotometric methods of direct determination of proteins in the presence of nucleic acids are very inaccurate. This is due to the fact that the amount of protein is measured from the absorption of light at wavelengths which are also partially absorbed by nucleic acids [619-621]. Therefore, the protein content of cells has been recently determined from the difference between the total weight (proteins + nucleic acids) and the content of nucleic acids [56, 468, 745, 864, 954, 955]. The total weight of proteins + nucleic a acids is found by means of interference microscopy, and the amount of nucleic acids determined from the absorption of ultraviolet light in the 260-265 mμ range. Proteins do not interfere in this case, since the absorption coefficients of proteins in this wavelength range is much lower than the absorption coefficients of nucleic acids [620, 740]. /127

Table 29 shows the concentrations of nucleic acids and proteins in the drops, found by using the indicated methods. Analysis of the data of Table 29 leads to certain conclusions:

1. The protein concentration of the drops as compared to the initial solutions from which the drops were obtained increases by a factor of tens and hundreds, and the greatest concentration is observed in clupein — RNA and clupein — DNA systems. The nature of the changes is the same as in the case of the total concentration of substances and concentration of nucleic acid.

2. In the histone-RNA system, it is obvious that the increase in the size of the drops is accompanied by a decrease in the protein concentration of the drops.

Table 29

PROTEINS AND NUCLEIC ACIDS IN COACERVATE DROPS

Diameter 10^{-4} cm	Volume 10^{-11} cm^3	Concentration, %		$\frac{(C)_{drop\ protein}}{(C)_{solution}}$*
		Nucleic acid	Protein	
		Histone - RNA		
4,0	33,5	8,9	24,1	58
5,5	87,1	4,0	7,4	18
8,0	268	2,5	5,5	13
		Histone - DNA		
6,0	113,1	11,2	14,8	21
7,0	179,5	4,1	5	11
		Clupein - RNA		
2,0	4,2	9	38	458
		Clupein - DNA		
3,0	14,1	8,3	59,6	718
4,0	33,5	7,8	51,2	628
5,0	65,4	6,7	40,3	486

*$(C)_{solution}$-concentration of protein in the solution from which the drops were formed.

3. If the ratio of protein to nucleic acid in the drop is considered, it is seen that the protein predominates over nucleic acid in most cases. In systems including gelatin + gum, the ratio of nucleic acids to the carbohydrate — protein is relatively low, 3-6%. In coacervates including alkaline proteins, the ratio of nucleic acid to protein amounts to 12% and more. There are drops in which there is 50% protein and 50% nucleic acid.

Coacervate systems contain drops with different relative proportions of protein and nucleic acids, and this ratio can be the same as in cell nuclei and ribosomes. Ribosomal RNA amounts to 80-90% of the total RNA of the cell [80, 361, 397].

The ribosomes of liver cells contain 40% RNA and 60% protein, and the ribosomes of E. Coli, conversely, 40% protein and 60% RNA. The ribosomes of reticulocytes consist of 50% protein and 50% RNA.

In coacervate drops of histone — RNA, which are much larger than ribosomes (5.5 mμ in diameter), thre was 65% protein and 35% RNA.

In organisms, various cells and nuclei having an internal structure are constantly encountered.

The nucleic acids in cells and subcellular structures are unevenly distributed in certain parts. Therefore, the study of the distribution of nucleic acids in structural coacervate drops of different forms is of great interest. /128

Distribution of Nucleic Acids in Drops. The distribution of nucleic acids in coacervate drops differing in shape and structure was studied in the same coacervates as those used for measuring RNA and DNA in globular coacervate drops [128-130, 672].

Method. The coacervates were obtained from histone — DNA, histone — RNA, clupein — DNA, and clupein — RNA.

The nucleic acids were determined under an MUF-4 ultraviolet microscope in the Kheysin Laboratory of the Cytology Institute of the Academy of Sciences (Leningrad) in cooperation with Miss Bukhman, on the staff of the Institute. A somewhat modified model of MUF-5 has now been put on the market. A detailed description of the design of the instrument is given in the paper of Bakharev et al. [24].

The MUF-4 ultraviolet microscope is a complex universal device which can be used to carry out spectroscopic studies in both solutions and cells by using different wavelengths of visible and ultraviolet light. The deisgn of the MUF-4 is based on the principle of the cytospectrophotometer, but it has essential modifications which permit the measurement of the content of substances, including nucleic acids, in various structures. This is done by automatically recording the absorption of the light rays (by scanning) in the form of a distribution curve which is then used to calculate the optical density and quantity of nucleic acids. During the recording, the microscope stage is moved together with the specimen toward the light beam which acts as the probe. Depending on the size of the object, a 1 or 2 μ probe can be employed. Consequently, measurements of densities can be made only in droplets no less than 1 μ in size, since otherwise, not only the droplet but also the equilibrium liquid surrounding it would be trapped. By passing the light beam through different portions of the droplet. One can obtain a complete picture of the distribution of nucleic acids in the droplet. In the MUF-4, optical densities from 0.2 to 1.2 are measured. Such values are in most cases characteristic of biological specimens.

In a quantitative determination of nucleic acids, in addition to the average optical density, it is necessary to know the size of the drop, which is calculated from photographs obtained in the same instrument. Drops were photographed in both visible and ultraviolet light in order to be able to compare the structure of the drop with the distribution of nucleic acids therein. The distribution curves were recorded only in ultraviolet light with λ equal to 265 mμ.

The calculation of the average optical density from the distribution curve can be explained by using the following example. Figure 51 a shows a curve of the recording of densities in a droplet having a uniform (diffuse) distribution of nucleic acid. The light probe traverses the drop in the direction indicated by the arrow. The upper point of the curve corresponds to the passage of rays along the diameter of the drop, where the rays are absorbed to the greatest extent, passing through the largest amount of substance. To the right of the curve are recorded standard attenuators (screens) corresponding to definite values of optical densities. Usually, no less than two such recordings are made, and then for each recording calibrated curves are plotted for the next reading of the average optical density of the structure as a whole. To this end, the height in centimeters at which each screen is located, measuring from the zero line of the recording, is laid off along the abscissa, and the density of each screen is laid off along the ordinate (in our work, the screen had densities of 0.3, 0.7 and 1.16 in all cases). A planimeter is then used to determine the area occupied under each curve or under its individual portions, and the calculated value is divided by the length of the base of the curve. The value obtained in centimeters is laid off as the abscissa along

/129

the calibration curve, and by finding the corresponding point on the ordinate axis, the average optical density is calculated. The amount of nucleic acids is calculated from the Lambert-Bouger-Beer from the formula

$$Q = \frac{DSd}{XA},$$

where Q is the amount of the substance in g; D is the average optical density, S is the area in cm^2, X is the extinction (absorption) coefficient, A is the distance travelled by the rays in the absorbed layer in cm, and d is the thickness of the specimen in cm.

Since the equilibrium liquid surrounding the droplet did not contain any nucleic acids, DFA was equal to unity, and therefore the content of nucleic acids was calculated from the dormula Q = DS/X. The absorption coefficients at 265 mμ for RNA and DNA were very similar and equal to 22,000 [5, 128, 619, 620]. This value is adopted in reading the optical density in the system of common logarithms in which the screens were calibrated. It should be noted that quantitative measurements of nucleic acids in individual structural formations inside the drops give a relative idea of the content of RNA and DNA in some cases. Errors may arise when the rays traverse not only the structure studied but also neighboring areas. For instance, before reaching a vacuole, the rays also pass through a layer of substances surrounding the vacuole. For this reason, data on the content of nucleic acids in vacuoles are usually high, particularly if the vacuole is small as compared to the overall size of the drop and if it is located at the center of the drop.

The error of the determinations made by using this method amounts to an average of 3-5% of the value being determined. Some of the results of average determinations are given in Table 30, and photographs of the drops in ultraviolet light and optical density distribution curves taken from these photographs are shown in Fig. 51, B-D. The various areas in the drops and recordings of the curves, denoted by Roman numerals, correspond to the same numerals in the table for each drop.

The data of Table 30 lead to the following conclusions:

1. One and the same coacervate system may contain droplets with different distributions of nucleic acids.

2. The distribution of nucleic acids in the drops may be of two main types: homogeneous (diffuse) and heterogeneous (nonuniform).

The heterogeneous distribution is characterized by a great diversity in the distribution of nucleic acids in the drops. There are drops which simultaneously contain portions with a low content of nucleic acids (vacuoles), and average and high contents (lumps, rings surrounding vacuoles). Occasionally, the bulk of nucleic acids is located on structural parts of the drop which can be observed in visible light.

3. The greatest diversity in the distribution of nucleic acids is characteristic of coacervate drops from histone and DNA and histone and RNA. In these coacervates, the drops have the most diverse structure. Since the composition of coacervates /132
obtained from clupein includes the same nucleic acids, the diversity in the distribution of nucleic acids is apparently due to histone.

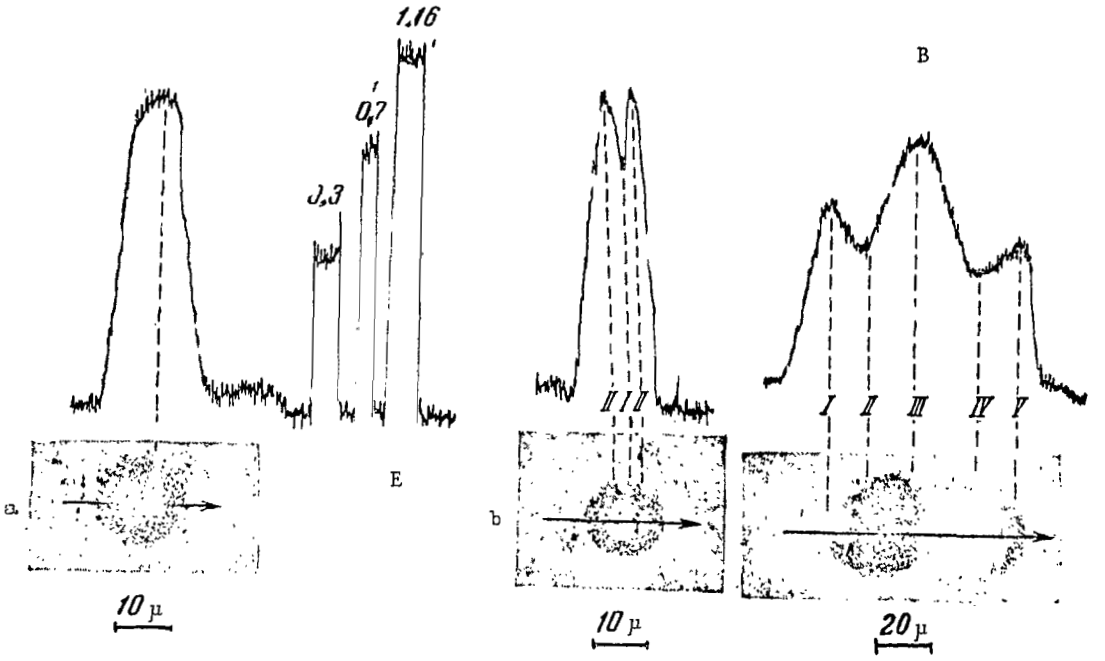


Figure 51. Distribution of Nucleic Acids in Coacervate Drops.
Arrow — Direction of Motion of Light Probe Through Drop.
A: a-coacervate drop from clupein — RNA; E-standard optical density; b coacervate drop from clupein — DNA, containing vacuole; I-vacuole; II-ring; B-coacervate drop from histone — DNA; I-ring; II-vacuole; III-boundary; IV-vacuole; V-ring; C: coacervate drops from histone — DNA (a, b); I-vacuole; II-ring; III-vacuole; IV-constriction; D: coacervate drop from histone — DNA; I-end of tail; II-lump; III-tail; IV-ring; V-vacuole; VI-head

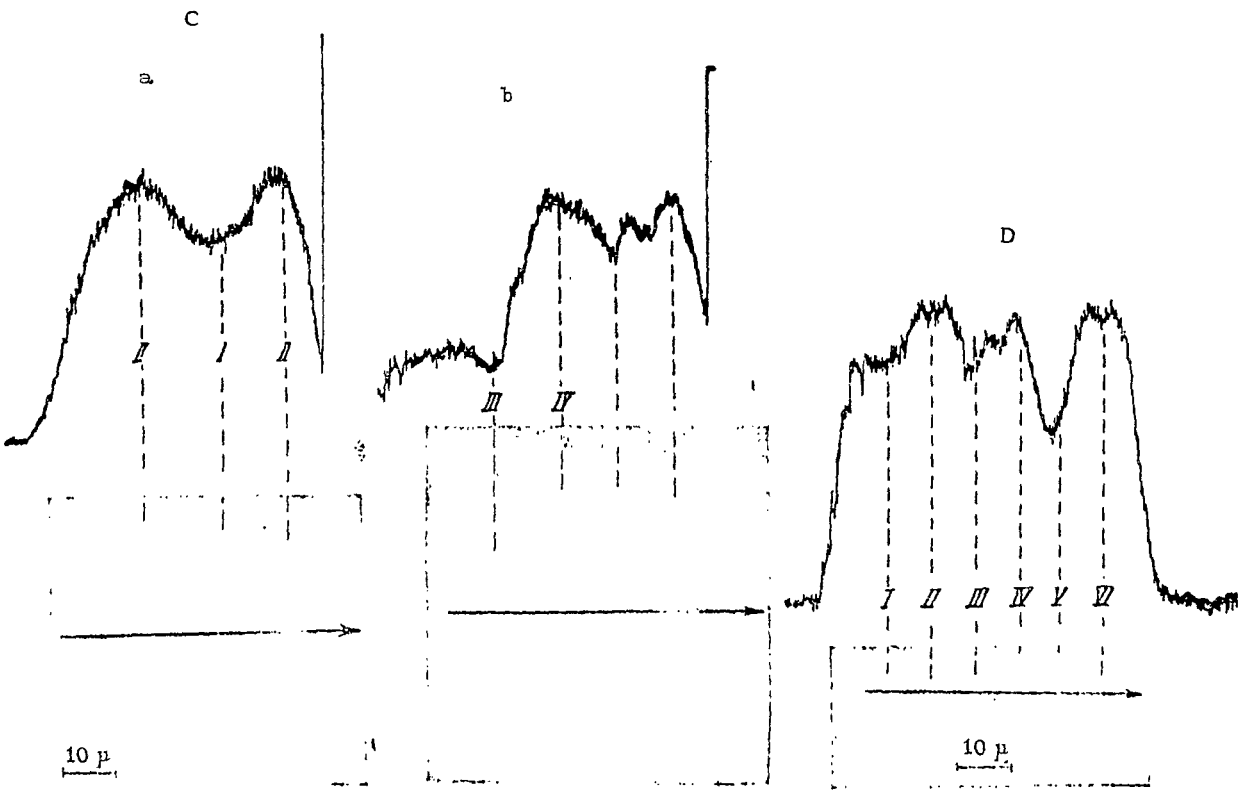


Fig. 51. (continued)

Table 30

DISTRIBUTION OF NUCLEIC ACIDS IN COACERVATE DROPS

Structure	Optical density	Area 10^{-6} cm^2	Nucleic acid 10^{-12} g
	Histone - DNA		
Drop	0.52	2.5	59.1
Drop	0.22	1.56	15.6
Part of drop:			
vacuole (I)	0.18	0.30	2.45
ring (II)	0.24	1.20	13.1
Drop	0.36	3.46	56.6
Part of drop:			
half-ring (I)	0.44	0.07	1.40
vacuole (II)	0.28	0.15	1.90
boundary (III)	0.50	0.91	20.7
vacuole (IV)	0.28	0.88	11.2
part of ring (V)	0.30	1.06	14.45
up to the boundary of ring	very low	0.37	–
Drop	0.45	5.42	110.86
Part of drop:			
vacuole (I)	0.38	1.08	18.65
ring (II)	0.47	2.23	47.64
vacuole (III)	0.22	0.64	6.4
constriction (IV)	0.58	1.47	38.75
Drop	0.70	2.97	94.5
Part of drop:			
end of tail (I)	0.50	0.23	5.23
lump (II)	0.72	0.23	7.53
tail (III)	0.53	0.26	6.26
ring (IV)	0.86	0.43	16.8
vacuole (V)	0.36	0.93	15.21
head (VI)	1.0	0.87	39.54
	Histone - RNA		
Drop	0.56	4.04	102.83
Drop	0.45	5.16	105.54
Drop	0.49	4.77	106.24
Drop	0.37	1.92	32.29
Part of drop:			
ring (I)	0.40	0.83	15.09
vacuole (II)	0.27	0.39	10.53
constriction + tail (III)	0.415	0.63	11.88

Table 30 (cont.)

Structure	Optical density	Area 10^{-6} cm^2	Nucleic acid 10^{-12} g
	Clupein - DNA		
Drop	1.0	2.66	120.90
Drop	0.53	1.72	41.42
Part of drop			
ring (I)	0.59	1.53	41.03
vacuole (II)	0.28	0.19	2.42
	Clupein - RNA		
Drop	0.65	2.01	59.38
Drop	0.70	5.52	175.63
Drop	0.28	5.86	74.58
Part of drop			
large	0.28	5.73	72.92
small	0.13	0.12	0.71

In our view, the diverse distribution of nucleic acids not only in coacervate systems differing in chemical composition but also within the same system is an interesting fact from the standpoint of the possibility of selecting structures suitable for further evolution. /134

CHAPTER VI

ABSORPTION AND CONCENTRATION OF CHEMICAL COMPOUNDS BY COACERVATE DROPS FROM THE SURROUNDING MEDIUM

Coacervate drops differ in properties from the solutions from which they were formed. The drops acquire the ability to interact with the surrounding medium by absorbing various substances from it, i.e., to concentrate compounds, as a result of the penetration of these substances from the equilibrium liquid. This property can be demonstrated most clearly on dyes.

Low Molecular Compounds

The ability of coacervate drops to be dyed was established by Bungenberg de Jong, Bank, Feldman and others [383, 385, 439, 537, 591], who developed mainly the qualitative aspect of this phenomenon.

Dyeing is a complex process dependent on various factors (size of the particles of the dye, its solubility, the degree of penetration into the specimen, etc.) [317]. Different theories of dyeing exist. Most dyes are electrolytes and carry charges different in sign and magnitude depending on the reaction of the medium and chemical nature of the dye [170, 297, 630]. The interaction of the dye with solutions of high molecular compounds such as proteins, nucleic acids, and carbohydrates can be represented in some cases as chemisorption associated with a definite chemical reaction between the negatively and positively charged groups in the molecules. For example, protein solutions at the isoelectric point dye very slightly, whereas in the presence of a minimum amount of free charges, the dissociation of both acid and alkaline groups is depressed. There exists a method of determining the isoelectric points of proteins from their ability to bind dyes. /135

During the dyeing, the proteins in the coacervates studied behaved as polycations, since the pH of the coacervates is in a more acid range than the isoelectric points of the proteins.

In order to make a qualitative and quantitative study of this phenomenon, fluorescent and ordinary dyes were tested.

Fluorescent dyes — fluorochromes — are used for both diffuse dyeing and for various structures in cells [181, 314]. The advantage of fluorochromes consists in the fact that they cause a fluorescence of the specimen in minute concentrations without appreciably damaging it [228].

A study of fluorochromes in coacervate systems was carried out in the ultraviolet microscopy laboratory of the State Optical Institute im. S.I. Vavilov in Leningrad together with a collaborator at the Institute, Barskiy [129]. Aqueous solutions of the dyes acridine orange, aurophosphine, and euchrisine were added to coacervates consisting of serum albumin-gum, gelatin-gum, starred strugeon protamine-gum, [302]. sickle protamine-gum, clupein-gum.

The fluorochrome concentration per unit volume of each coacervate (1 ml) was 12-48· $\cdot 10^{-6}$ g.

The coacervates with the dye were placed in a glass cell 0.13 m thick on the stage of a luminescent microscope of special design [69]. The light source was an SVDSH-250 lamp. The drops were observed and photographed with objectives x 20 x 0.65 and x 40 · 0.65 at wavelengths of 300-450 mμ, separated by a light filter from the total radiation of the lamps.

Blank determinations of the luminescence of the coacervate without fluorochromes showed that the equilibrium liquid does not luminesce, and that the drops have a bluish-white light characteristic of the majority of protein substances, which once again proves that proteins concentrate in the drops.

In the dyed coacervates, the drops luminesced very brightly. Depending on the illumination time and dye content of the droplet, the color with acridine orange varied from reddish to yellow.

Aurophosphine gave a yellow luminescence, and euchrisine a green one. The field of view remained dark. There exists a definite limit of saturation of the drops with fluorochromes. In the presence of a considerable excess of fluorochromes, part of them are not abosrbed by the drops and remain in the equilibrium liquid. In this case, the field of view also luminesces.

Hence, fluorochromes are absorbed by drops from the equilibrium liquid, and as a result, their concentration in the coacervate drops increases markedly. In this respect, fluorochromes behave like ordinary dyes. /136

In most cases, coacervates containing alkaline proteins extensively absorbed toluidine blue, i.e., precisely the dye employed for dyeing cell nuclei. The composition of nuclei includes alkaline proteins of the type of histones [25, 633, 637, 638, 832].

Coacervates with acid proteins adsorb eosin, methylene blue and neutral red equally well, which are most frequently used for dyeing cell protoplasm [239].

It is interesting to note that the multicomponent coacervate of histone-gum-glucose-1-phosphate-phosphorylase and starch at pH 6.2 was best dyed with carmine, and the two-component coacervate of histone — gum at pH 5.8-6.0 was best dyed with eosin.

Despite the similar pH values and certain common components — protein and carbohydrate, the coacervates differed from one another. This difference obviously also depended on other compounds entering into the composition of the multicomponent coacervate.

A quantitative determination of the dyes was performed in individual globular unstructured drops. The most suitable system for this purpose was a coacervate of gelatin — gum, to which methylene blue or neutral red was added.

The absorption time of the dye depends on the volume of the coacervate and amount of dye taken. For example, for 0.25 ml of coacervate consisting of gum — gelatin and 0.15-0.25 ml of a 10% aqueous solution of methylene blue, the absorption time at 16° was 30-40 min. [120, 248]. The dyeing process is conveniently followed by means of

motion picutre films. Such films were taken during the study of absorption of various dyes by protein-carbohydrate [120] and alkyl amide coacervate drops [224].

In ref. [129], we presented photographs of drops before the addition of methylene blue, then after the introduction of the dye, when the entire field of view was uniformly dyed a blue color, and finally at the instant when the entire dye was absorbed by the drops. It should be emphasized that some of these dyed preparations of dried drops have been preserved since 1949, and that if water is dropped on them, the precipitates swell up and drops reappear.

The amount of dye absorbed by individual drops was found by measuring the optical density in the visible range in a cytospectrophotometer [299, 365].

For drops with methylene blue, the optical density was measured at λ equal to 514 mμ, and with neutral red, at 660 mμ. The optical density and content and concentration of dyes in the drops were calculated from formulas (5-6) (cf. 122). /137

As follows from the formulas, in order to calculate the amount of dye adsorbed by a drop, it is necessary to know the absorption coefficient of the dye. The absorption coefficients of dyes may change depending on the dye preparation and also on the conditions of their measurement: pH, concentration of the dye itself, degree of aggregation of the molecules, etc.

In addition, as reported by various authors [99, 170, 404, 630, 809], the chemical formulas of methylene blue and neutral red differ. For all these reasons, appreciable discrepancies in the absorption coefficients of the dyes are observed. We therefore determined these coefficients on a cytospectrophotometer and SF-4 spectrophotometer. The molar coefficient of neutral red E = 4700, and for methylene blue, E = 27,000. The values obtained are close to the data reported in the literature [244].

In our calculations, instead of molar coefficients, it was more convenient to use the so-called percent coefficient (K):

$$K = \frac{D}{c \cdot d}, \qquad (1)$$

where c is the percentage, d is a 1-cm thickness of the layer, and D is the optical density [96].

The error in measurements of optical density in drops dyed with neutral red was 2-3%, and with methylene blue, 5-7% of the value being determined.

Since the main purpose was to explain not the absolute concentration of dyes in the drops but the ability of the drops to concentrate the dye as compared to the initial solutions, such discrepanceis in the determinations for methylene blue and neutral red were of nor real importance. In each coacervate system, an average of about 100 drops were measured. Part of the results are given in cumulative Table 31 and in Fig. 52.

On the graphs, the broken line denotes the content of the dye in the drops, providing that the concentration of the dye is the same in all the drops. The concentration found in the smallest droplet was taken as a constant value. The solid line indicates the content of dyes in drops found by experimentally determining the the concentration in each drop. /138

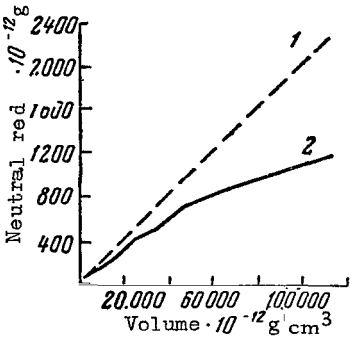


Figure 52. Coacervate Drops from Gelatin - Gum + Neutral Red.
1-content of dye in drops calculated at a constant concentration measured in the drop of the smallest diameter;
2-content of dye obtained experimentally.

The data of Table 31 and Fig. 52 indicate that differences in the percent content of dyes between small and large drops are relatively slight. This is due to the small amount of dyes added. Drops can be obtained in which the concentration of the dye is high, but in this case a part of the dye is not absorbed and remains in the equilibrium liquid. In addition, when the drops are highly saturated with the dyes, the opitical density increases markedly, which affects the accuracy of the results.

The content of the dye increases simultaneously with the volume of the drop, and the concentration per unit volume decreases, i.e., the same picture is observed as in the case of the total weight of the substances and nucleic acids in the drops.

As a result of absorption of the dye in the drops, its concentration increases tens of times as compared to the total content of the added dye per unit volume of the coacervate. For example, the concentration for a solution of methylene blue was 5 mg %, and in a drop $16 \cdot 10^{-4}$ cm in diameter it increased to 270 mg %.

The concentrating of dyes may be regarded as the ability of the drops to absorb low molecular heterocyclic compounds from the medium surrounding them.

Amino Acids. Compounds from which all proteins are made up are extremely important. The study of the behavior of amino acids in coacervate systems is of interest because they are significant not only for organisms but also for understanding the origin of life on earth. The possibility of formation of amino acids has been demonstrated in cell-free systems by means of enzymes and also abiogenically [8, 22, 23, 278, 279, 294, 340, 341, 402, 437, 810, 811].

Some data were obtained by Liebl. He demonstrated the ability of the gelatin - gum coacervate to absorb an added mixture of amino acids: alanina, valine, phenylalanine, histidine, lysine, arginine, cysteine, aspartic acid and also glutathione tripeptide [773]. The total amino acids were determined by dyeing with ninhydrin in the coacervate layer and in the equilibrium liquid. In 0.1 ml of coacervate layer corresponding to all of the drops, $37 \cdot 10^{-6}$ g was found, and $18 \cdot 10^{-6}$ g was found in the same volume of equilibrium liquid [211]. Thus, a certain concentration of amino acid took place in the drops.

The distribution of individual amino acids, tyrosine and tryptopah was studied in protein — carbohydrate, protein and protein — lipid coacervates [146]. The method of preparation of the coacervates is described below: /140

Gelatin — Gum Arabic Coacervate. 0.67% solutions of gum arabic and gelatin preheated to 42° and prepared with 1/15 M acetate buffer in a solution with pH 3.8-4.0 were combined in the ratio of 5:3. The volume of the entire coacervate was 8 ml, and the drop fractions were 0.2 ml.

Clupein — Gelatin Coacervate. Aqueous solutions of 0.67% gelatin and 1.5% clupein

Table 31

CONTENT OF DYES IN INDIVIDUAL COACERVATE DROPS OF GELATIN — GUM

No.	Diameter 10^{-4} cm	Volume 10^{-12} cm^3	Content, 10^{-12} g	Concentration, (C)%	$\frac{C_{drop}}{C_{solution}}$*
		Neutral red			
1	12	904.39	4,97	0.55	34
2	28	11489.13	54.0	0.47	29
3	32	17149.95	56.60	0.33	20
4	36	24418.58	63.5	0.26	16
5	40	33496.00	113.9	0.34	21
6	44	44591.55	138.2	0.31	19
7	52	73607.46	198.7	0.27	17
		C_r = 0,016%; there is no dye in the equilibrium liquid			
8	20	4187.0	87.1	2.1	33
9	24	7285.14	80.1	1.1	18
10	28	11489.13	217.14	1.9	29
11	32	17149.95	274.4	1.6	25
12	36	24418.58	434,65	1.8	28
13	40	33496.0	535,94	1.6	25
14	40	38496.0	509,14	1.5	23
15	44	44591.55	713,46	1.6	25
16	48	57864.34	810,1	1.4	22
17	48	57864.34	839,03	1.5	23
18	60	113049	1243,53	1.1	17
		C_r =0,064%; 0.001%; of dye was found in the equilibrium liquid			
		Methylene blue			
19	16	2143.7	5.7	0.270	54
20	20	4187.0	8.8	0.210	42
21	24	7235.1	10.1	0.140	28
22	28	11489.1	12.6	0.110	22
23	28	11489.1	16.1	0.140	28
24	36	24418.6	41.3	0.169	34
25	40	33496.0	47.0	0.140	28
26	40	33496.0	53.6	0.160	32
27	50	65401.0	111.2	0.170	34
28	56	91904.6	177.6	0.190	38
29	60	113049.0	180.9	0,160	32
		C_r =0.005%; there is no dye in the equilibrium liquid			

*($C_{solution}$)-concentration of dye in the solution from which the drops were obtained.

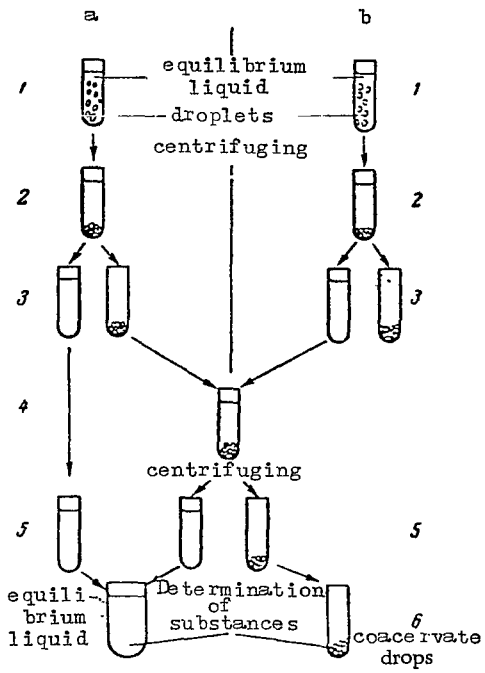


Figure 53. Diagram of the Determination of Various Substances in Coacervates
a-coacervate+added substance; b-coacervate without added substance; 1-coacervates; 2-3-separation into fractions of drops (deposit on the bottom) and equilibrium liquid; washing of the drops off the introduced substance by means of equilibrium liquid not containing this compound; 5-combination of the wash and equilibrium liquids containing various compounds; 6-quantitative determination of the substance in the drops and in the equilibrium liquid

sulfate preheated to 50° were combined in the ratio of 1:1 and alkalized with a 0.1 M solution of NaOH to pH 8.6-8.8. The entire volume of coacervate with amino acids was 2 ml, and the drop fraction was 0.1 ml.

Oleate — Gelatin Coacervate. To 2 ml of a 1 N borate-phosphate buffer solution with pH 8.4 preheated to 42° were added 0.4 ml of a 3% aqueous solution of gelatin and 0.6 ml of a 0.1 N solution of potassium oleate. The latter was obtained by neutralizing oleic acid with an equivalent amount of KOH. The volume of the entire coacervate was 3 ml, and the drop fraction was 0.3 ml. Different amounts of radioactive tyrosine tagged with C_{14} and tryptophan [50, 190] were added to the coacervate.

During the experiments, the preservability of the drops was checked under the microscope. The distribution of amino acids between the drops and the equilibrium liquid was analyzed by using the procedure shown in Fig. 53.

The course of the analysis is as follows:

1. Two coacervates are taken: one with an amino acid and the other without it. Both coacervates are incubated for 15 min.

2. Each coacervate is separately centrifuged at 1500-2000 rpm for 3 minutes with fast cooling to 0°. Under these conditions, the droplets deposit to the bottom and walls of the test tube without fusing together (in all subsequent cases the centrifuging is carried out in the same manner).

3. After the centrifuging, the equilibrium liquids are transferred into separate test tubes.

4. Droplets with the amino acids are washed off the equilibrium liquid containing excess amino acid. To preserve this system, the washing is done by using the equilibrium liquid of the coacervate without the amino acid. After the washing, the equilibrium liquid is separated by centrifuging.

5. The amino acids are determined in the washed coacervate drops and in the combined equilibrium liquids (from the coacervate with the amino acid and without it) and also in the initial coacervate with the amino acid and in the blank experiments. The blanks used were coacervates without amino acids. This procedure was used to study coacervates to which amino acids were added both before and after the formation of drops. The amount of drops was determined by modified colorimetric methods,

tyrosine by Bernhardt's method, and tryptophan by the method of Block and Bolling [13]. The colorimetry was performed on an SF-4 spectrophotometer or an FEK-52 photoelectrocolorimeter at the following wavelengths: 500 mμ for tyrosine and 420 mμ for tryptophan. The error of the methods employed was 5-10% of the value being determined.

In all the coacervates, gelatin containing tyrosine is the mandatory component. Depending on the method, the total amino acid or only a part thereof is observed [13]. In the latter case, hydrolysis of the gelatin is carried out when the total amino acid is determined. In addition, depending on the source and method of isolation of gelatin, the latter may contain different amounts of tyrosine, and sometimes also tryptophan. The content of the amino acids tyrosine and tryptophan of the gelatin was checked before and after hydrolysis. The data were found to be quite similar. For example, prior to hydrolysis, 0.378% tyrosine and 0.179% tryptophan were found in our preparation, and after hydrolysis, 0.382% tyrosine and 0.18% tryptophan. For this reason, all the determinations of amino acids in coacervates were subsequently carried out without hydrolysis [41, 146, 219].

The order of introduction of the amino acid into the coacervate before or after the formation of drops did not affect the distribution of amino acids between the drops and the equilibrium liquid.

Results of experiments on the introduction of different amounts of amino acids — radioactive and ordinary tyrosine and also tryptophan are shown in Tables 32 and 33 and in Fig. 54.

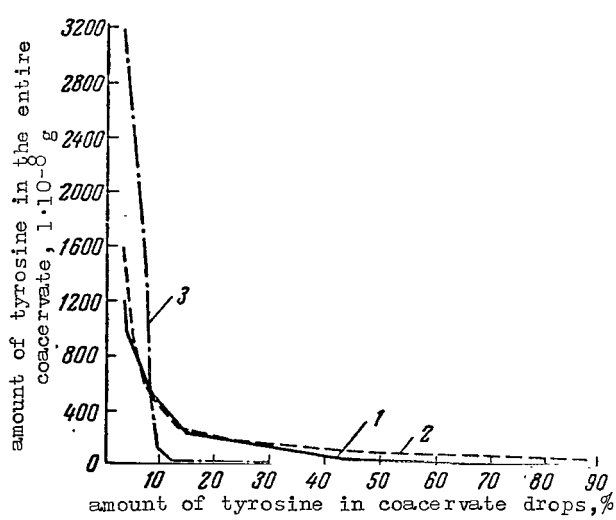


Figure 54. Distribution of Tyrosine in Coacervates.
1-gelatin - gum; 2-gelatin - clupein; 3-K oleate - gelatin

The data of Tables 32 and 33 and the graph of Fig. 54 show that the less amino acid has been introduced into the coacervate, the greater part of its is present in the coacervate drops. This may be explained by the fact that there exists an upper limit of saturation of the drops with amino acids beyond which the amino acid content of the drops remains unchanged, despite the increase in the amount of amino acids in the system. This limit depends first of all on the nature of the amino acid and on the protein component, and secondly on the pH of the other components of the coacervate. Thus, for example, when the drops are completely saturated with the amino acids in the gum arabic — gelatin system at pH 3.8-4.0, for every 23 molecules of tyrosine present in the gelatin contained in the drops, there are 10 molecules of tyrosine added to the system, and for every 10 molecules of tryptophan as such, there are 13 molecules introduced from the outside. As the pH increases from 8.6 to 8.8 and the composition of the coacervate (clupein — gelatin) changes, the amount of amino acids increases. Thus, for 10 molecules of tyrosine and 10 molecules of

tryptophan of the gelatin of coacervate drops, there are 24 molecules of tyrosine and 28 molecules of tryptophan introduced from the outside, and, as was shown by additional experiments, the amino acids are bound only to gelatin, not to clupein. At the same time, at pH 8.4, only three molecules of added tyrosine per molecule of tyrosine of the coacervate drop fraction pass into the oleate — gelation coacervate drops. /142

The type of bonding between the amino acids and the component parts of coacervates in the drops has not been studied thus far.

The concentration of amino acids in the drops is tens of times as high as in a unit volume of the entire coacervate and hundreds of times as high as in the equilibrium liquid. Thus, for example, the tyrosine content of the equilibrium liquid was 0.005%, and 1.45% in the drop fraction.

Thus, the distribution of amino acids between the drops and the equilibrium liquid depends on the chemical nature of the amino acid and the composition of the coacervate.

Sugars. Different sugars and also polysaccharides could have formed abiogenically, like amino acids and other compounds [618, 792, 799, 821, 886, 887]. Troshin has made a very thorough study of the distribution of galactose and sucrose in the coacervate of gum arabic and gelatin. The content of sugars in the coacervate layer and equilibrium liquid was determined 15 hours after their addition to the coacervate. Experiments showed that at low sugar concentrations, the bulk passes into the coacervate layer. Only a small part remains in the equilibrium liquid. In this case, the ability of the coacervate layer to sorb, i.e., concentrate the sugar, is of prime importance. When the concentration of the initial solutions of sugars is substantially increased (beginning with 10% solutions), the saturation limit of the coacervate layer is reached, and the excess accumulates in the equilibrium liquid. At this point, however, a major part begins to be played by the decrease in the solubility of the substances from which the coacervate was obtained. As a result, there is at first observed a decrease in the volume occupied by the coacervate layer, and in the end the coacervate disintegrates. Phenomena of this kind take place in live specimens as well, for example in the distribution of arabinose, galactose, and sucrose between frog muscles and the ambient Ringer solution, and in the distribution of glucose between erythrocytes and blood plasma. A change in the volume of erythrocytes may take place under the influence of not only sugars but also mineral salts. For example, as the NaCl concentration increased from 0.91 to 5%, the volume of an erythrocyte decreased and amounted to 62.9% of the initial value [375-378, 934]. /143

Mineral Salts. Salts are regulators of many enzyme reactions. Because of their small molecule and high dissociation, they readily penetrate into various systems and leave them.

Bungenberg de Jong and his school thoroughly studied the problem of the effect of salts on coacervates, mainly Ca, K, and Na. Voorn showed that on the whole, in all coacervate drops from gum-gelatin-chloride, the KCl content increases by a factor of 1.08-1.12 as compared to the equilibrium liquid. No substantial concentration of the salt is observed. However, in the author's view, even this quantity is of considerable significance, since potassium plays a major part in physiological processes [953].

Liebl studied the sorption of radioactive copper in coacervates at pH 3.95. He observed a somewhat higher Cu-64 content in the deposit corresponding to coacervate /145

Table 32

DISTRIBUTION OF TYROSINE IN COACERVATES*

Coacervate system	Coacervate 10^{-4} g	Drops		Equilibrium liquid		Ratio of amino acid of drops to amino acid of gelatin, %
		10^{-6} g	% of total content	10^{-6} g	% of total content	
			Tyrosine			
Gelatin - gum arabic	33.0	29.0	87.90	4.0	12.10	29.00
	55.0	35.0	63.60	20.0	36.40	35.00
	67.5	30.0	44.40	34.0	50.40	30.00
	110.0	42.0	38.20	68.0	61.80	42.00
	135.0	46.0	34.10	88.0	65.10	46.00
	270.0	40.0	14.80	228.0	84.40	40.00
	540.0	46.0	8.50	470.0	87.00	46.00
	1200.0	42.0	3.50	115.2	96.00	42.00
Cluepein - gelatin	50.0	44.0	88.00	6.0	12.00	244.00
	100.0	45.0	45.00	55.0	55.00	250.00
	200.0	44.0	22.00	156.0	78.00	244.00
	300.0	40.0	13.30	260.0	86.70	222.00
	500.0	44.0	3.80	456.0	91.20	244.00
	600.0	42.0	7.00	558.0	93.00	233.00
	800.0	42.0	5.30	758.0	94.70	233.00
	1000.0	48.0	4.80	952.0	95.20	266.00
	1500.0	45.0	3.00	145.5	97.00	250.00
Oleate - gelatin	10.0	3.2	32.00	6.8	6.80	9.24
	20.0	5.9	29.50	4.1	71.50	17.10
	50.0	6.0	12.00	44.0	88.0	17.39
	100.0	10.5	10.50	89.5	89.50	36.41
	250.0	20.6	8.24	229.4	91.76	59.53
	500.0	39.8	7.90	460.2	92.10	105.47
	975.0	74.0	7.59	901.0	92.41	213.59
	1000.0	75.5	7.55	924.5	92.45	215.32
	1500.0	107.2	7.15	1392.8	92.75	309.83
	3280.0	110.0	3.35	3170.0	96.65	317.92
			Tryptophan			
Gelatin - gum arabic	200.0	11.0	5.50	189.0	94.50	16.00
	2100.0	90.0	4.30	201.0	95.70	143.00
	3000.0	84.0	2.30	351.6	97.70	133.00
Clupein - gelatin	300.0	28.0	9.30	27.2	90.70	233.33
	1500.0	34.0	2.27	146.6	97.73	232.50
	3000.0	34.0	1.18	266.0	98.87	232.50

*All the figures in the table are given after subtracting tyrosine and tryptophan, which are present in gelatin alone which enters into the composition of the coacervate drops. The amount of tyrosine in the gelatin of coacervate drops in the gelatin - gum arabic system is $100 \cdot 10^{-6}$ g, in the coacervate from gelatin and clupein - $18 \cdot 10^{-6}$ g, and in the oleate - gelatin coacervate, $34.6 \cdot 10^{-6}$ g. The content of tryptophan in the gelatin - gum coacervate was $47.3 \cdot 10^{-6}$ g, and in the gelatin - clupein coacervate, $8.5 \cdot 10^{-6}$ g.

Table 33

DISTRIBUTION OF AMINO ACIDS
IN COACERVATES PER UNIT VOLUME (1 ml)

Coacervate	Amino acid	Amino acid			Ratio of amino acids of drops to amino acids	
		In coacervate 10^{-6} g	In equilibrium liquid 10^{-6} g	In coacervate drops 10^{-6} g	coacervates	equilibrium liquid
Gelatin - gum	tyrosine	4.12	0.51	145.00	35.19	284.30
	tryptophan	25.00	24.30	55.00	2.20	2.30
Clupein - gelatin	tyrosine	25.00	3.16	440.00	17.60	139.24
	tryptophan	150.00	143.50	280.00	1.86	1.95
Oleate - gelatin	tyrosine	3.33	2.52	10.70	3.20	4.23

drops as compared to the equilibrium liquid. However, even in this case there is no high concentration of copper in the drops [235, 670, 773].

Iron, which has a catalytic effect, is distributed even more extensively, but its activity increases by a factor of several hundred, when it is present in the composition of the prosthetic groups of cytochromes, oxidation enzymes and catalase; the effect of the latter in coacervates will be described in Chapter VII. An $FeCl_3$ solution was added to the gum - gelatin coacervate at pH 3.8-4.0 in order to elucidate the ability of iron to concentrate in the drops. Compounds with trivalent iron absorb ultraviolet light. The coacervate with iron was photographed in visible light and in ultraviolet light with wavelengths of 250-280 mμ.

The pictures obtained clearly indicate that iron accumulates predominantly in the drops, but it is also present in the equilibrium liquid. The distribution of iron has the same character as in other salts [129].

It follows from these data that coacervate drops absorb different low molecular compounds, thus substantially increasing their concentration [129].

High Molecular Compounds

As an example of the absorption of high molecular compounds, Table 34 gives the results obtained by Liebl, who studied the sorptive properties of a four-component coacervate of gum arabic, gelatin, histone and RNA at pH 4.0 and 37-40°. The total volume of the coacervate was 5.5 ml; the volume of the drop fraction was 0.1 ml [211].

It is apparent from the data of Table 34 that coacervate drops absorb and concentrate high molecular compounds, and that the content of the sorbed substances in the drops is tens and hundreds of times as high as in the equilibrium liquid.

However, it is difficult to compare the absorptions of different compounds, since different initial amounts of substances were added to the coacervate. It is quite probable that the saturation limit takes place at a lower content of the compound than was taken for the experiment.

Table 34

DISTRIBUTION OF HIGH MOLECULAR COMPOUNDS
IN COACERVATES
(SORPTIVE PROPERTIES OF COACERVATES)

Compound	Content, 10^{-4} g/l ml		drops / equilibrium liquid	Added to coacervate 10^{-3} g
	drops	equilibrium liquid		
Myoglobin	10500	260	40	25000
Hemoglobin	13000	220	59	25000
Serum albumin	7500	85	88	12500
Papain	70	0.5	140	5
Ribonuclease	4	0.02	200	0.3

In this case, a part of the added compound remains in the equilibrium liquid and the ratio $\frac{\text{drops}}{\text{equilibrium liquid}}$ decreases.

Such a phenomenon is most pronounced in the case of hemoglobin and myoglobin. At the same time, the highest ratio is observed for papain and ribonuclease. The total content of these compounds in the coacervate was the lowest. /146

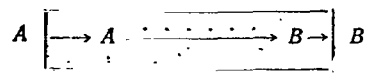
The ability of drops to concentrate both low molecular and high molecular compounds from the solution surrounding them was obviously of definite importance both for the subsequent increase in their complexity and further evolution, and for the separation of the substances in the protoplasm. A very important stage was the absorption of catalytically active compounds by the drops. Since life and its formation cannot be represented without catalytic reactions, the problem of the sorption and action of enzymes in coacervate systems is of prime importance.

Data on the absorption of enzymes and various substrates on which they act are presented in the next chapter.

CHAPTER VII

COACERVATES AND ENZYMES

A further increase in the complexity of coacervate drops and their improvement took place only when they interacted with the surrounding medium. However, if this interaction is limited to the absorption and concentration of substances from the surrounding solution, an equilibrium excluding any further complication of structure rapidly takes place. To prevent this from happening, chemical reactions should take place in the systems. In this case, the drops assume the properties of an open system in which a constant stream of a single direction takes place [49, 252-253, 264, 282, 300, 756] according to the diagram



where A is the substance penetrating the system; B is the reaction product capable of rediffusing into the external medium. For such a stream to occur, it is necessary that the reaction proceed in the system more rapidly than in the surrounding medium, since only then will there be a constant penetration of substance A from the medium and the diffusion of substance B from the system into the medium. An increase in the reaction rate in the coacervate drop may be caused by the concentration of substances A, removal of the reaction product into the external medium, and inclusion of catalyst in the drops.

The most active catalysts are protein-enzymes [95, 117, 126, 165, 282, 309]. For example, 1 g of the enzyme pepsin in 2 hours at 37° can cleave 50 kg of egg albumin. If instead of pepsin, 20% sulfuric acid used in laboratories for cleaving proteins is taken, a temperature $>100°$, 24 hours and 1 T of acid will be required.

At the present time, over 700 enzymes performing numerous transformations of substances in cells have been isolated. However, the detailed structure of most enzyme molecules has not yet been determined [154, 180, 242]. For this reason, the adopted classification of these compounds is rather functional and based on the ability of the enzymes to take part in chemical reactions of a definite type. According to this classification, enzymes are distributed into six categories: oxido-reductases, transferases, hydrolases, lyases, isomerases and ligases. /148

Oxidoreductases perform oxidation-reduction reactions connected mainly with processes of energy production (respiration, glycolysis, etc.); transferases carry individual radicals from one compound to another. Hydrolases split intricate compounds into simpler ones by means of water. Lyases rupture chemical bounds most frequently between carbon atoms in -C-C- without the participation of water. The activity of the isomerases is accompanied by a transposition of the double bonds or radicals inside the molecule. The ligases (synthesases) synthesize compounds by using the pyrophosophate bond of ATP [176, 867]. Reactions of similar type could have taken place before the appearance of life. However, in this case the role of catalysts was played by metals and also compounds of simpler structure than enzymes [35, 164].

The use of enzymes in coacervate systems is of interest not only from the standpoint of their high catalytic activity, but also for elucidating characteristics of enzyme reactions in coacervates in the case of separation of the substances in protoplasm in coacervate form.

Protoplasm belongs to liquid heterogeneous systems with many interfaces and separate structures providing for the separation of the substances and enzyme reactions [344, 345]. The formation of natural coacervate drops in protoplasm also causes an increase of heterogeneity.

Each droplet has an independent spherical interface at the boundary with the surrounding liquid. In addition, in structured drops, when the distribution of substances is uneven, vacuoles and clumps are formed, and additional surfaces appear.

Thanks to these properties, molecules of diverse compounds can concentrate in various areas without fusing together.

Thus, the presence of drops gives rise to conditions for heterogeneous enzyme catalysis in liquid structures. Obviously, the action of enzymes in such systems should differ from reactions in homogeneous solutions.

For a long time it was not known whether enzymes could act in artificial coacervate systems. The first studies along these lines which gave positive results were published in 1955 [148, 261]. /149

The modeling of enzyme processes in coacervates involves many difficulties. Enzymes are most active in a definite pH range. Each coacervate system is also formed at a rigorously defined hydrogen ion concentration.

For this reason, it is necessary to match the pH of the action of an enzyme with the pH of the existence of the coacervate [113]. Different coacervates can be obtained in the 1.2-11.2 pH range [129]. The multitude and diversity of the chemical composition of coacervates practically always makes it possible to select a suitable model for any enzyme reaction. Usually, the behavior of enzymes in coacervates is studied as a function of the pH, temperature, time, chemical composition of the coacervates and also the concentration of the enzyme and substrate on which it is acting.

The activity of enzymes in coacervates is compared to their action in buffer solutions. In addition, the distribution of enzymes, substrate and products of the reaction between the drops and the equilibrium liquid is investigated.

The separation of the coacervate into the fraction of drops (denoted by the word drop) and the equilibrium liquid is carried out by centrifuging at 2-0° for 5-10 min at 1500-3000 rpm. Under these conditions, the enzymes are practically inactive, and the droplets are well preserved, depositing on the bottom and walls of the test tube.

Further treatment of the fractions was carried out in accordance with the scheme shown in Fig. 53 and used for analyzing the distribution of amino acids in coacervates The degree of activity of the enzyme and the quantitative content of compounds present in the coacervate system are determined by methods specific for each of them.

It is most convenient to work with coacervates 2-9 ml in volume. In this case, 0.1-0.3 ml corresponds to the fraction of drops, and 1.9-8.7 ml corresponds to the equilibrium liquid.

Thus, drops occupy a slight volume as compared to the equilibrium liquid. Therefore, in order to compare different fractions, the results are given in terms of the same unit of volume, equal to 1 ml.

Of great importance is the nature of the substances from which the coacervates were formed, particularly the method of isolation of enzyme preparations. For this reason, the original studies give detailed data on the methods of isolation of the enzymes.

At the present time, the following enzyme reactions have been modeled in coacervates: 1) hydrolytic cleavage of polysaccharides, nucleic acids, proteins [148, 211, 261, 261, 262]; 2) synthesis of polysaccharides and plynucleotides [149, 151, 260, 263, 266]; 3) oxidation — reduction reactions, and also the cleavage of hydrogen peroxide by catalase [143, 327, 333, 334, 336]. /150

Among these reactions, the most accessible are processes of hydrolytic cleavage of natural polymers into low molecular compounds. Hydrolysis of starch accomplished with β and α-amylases has been known and widely employed for a long time. These enzymes were the first to be studied in coacervate systems.

Hydrolytic Reactions

Enzymes of α- amylase type hydrolyze polysaccharides with the participation of water into low molecular compounds — dextrins, disaccharides, and glucose. The chief products of the reaction are dextrins [675, 676]. To reproduce this reaction in a coacervate, use was made of α-amylase (α-1, 4-glucan-4-glucano-hydrolase 3.2.1.)* isolated from the culture liquid of the thermosphilic variant of Clostridium pasteurianum grown at 60° [45, 161]; the α-amylase had a high diluting and dextrinizing capacity and a low saccharification value of the starch [129, 304].

A temperature of 60° and pH of 5.5-5.9 were found to be the optimum values for the action of this preparation. However, the α-amylase retained its activity also at temperatures of 45-90° and pH of 5.0-9.0. Therefore, in order to study its action in the coacervate, coacervate drops were prepared consisting of soluble starch, protamine sulfate and gelatin. Such drops are formed in the system at pH 5.6-8.4 and a temperature of 50°.

The method of studying the action of α-amylase in the coacervates consisted in the following. To 0.4 ml of a 1% solution of soluble starch were added 0.4 ml of a 0.5% solution of protamine sulfate, 1.2 ml of a 0.67% solution of gelatin, and 0.1 ml of a solution of a α-amylase containing 0.2 mg of enzyme. All the solutions were first heated to 50° and combined in the indicated order. The mixture was then alkalized with a 0.01 N solution of NaOH to pH 7.0, at which the coacervate drops were formed. At pH 7.0, a better formation of the drops was observed, since in a more acidic range, the drops were found to be too fine. In addition, in a more alkaline reaction, iodine does not produce the characteristic color with starch and products of its breakdown, i.e., dextrins; the action of α-amylase on the starch was determined from the different color of the drops with the iodine solution.

Depending on the degree of hydrolysis of the starch, the color of the drops gradually changed, starting with blue (starch), then turning violet (amylodextrins), then red (erythrodextrins), and finally, yellow (achrodextrins). In blank determinations with the inactivated enzyme, the color of the drops remained bluish-violet in the course /151

*For each enzyme, the monograph indicates in parentheses its systematic name according to the classification of enzymes adopted in 1962 [176].

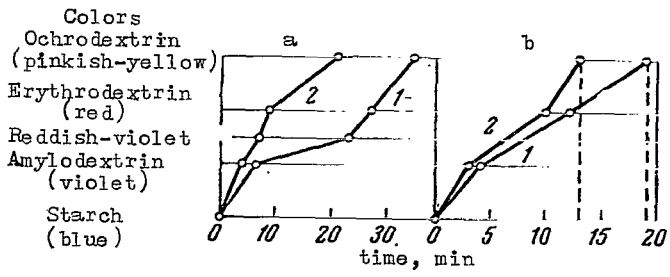


Figure 55. Decomposition of Starch under the Influence of the Enzyme α-amylase in Protein Coacervate.
a: 1-α-amylase + coacervate (pH 7.0); 2-α-amylase + soluble starch (pH 7.1); b: 1-α-amylase, gelatin, protamine (pH 5.5); 2-α-amylase, soluble starch (pH 5.2).

of the entire experiment. Color photographs of the drops, dyed different colors, are shown in the corresponding original study [261]. The rate of change of the color in the coacervate system is shown in Fig. 55.

The data cited show that in the coacervate system the rate of cleavage of starch, particularly to the final stage of achrodextrins, is approximately 1.5 times slower than in an aqueous solution. The coacervate components themselves - gelatin and protamine — were found to decrease the activity of the enzyme somewhat. Unfortunately, it is impossible to check the action of the enzyme on soluble starch in the presence of gelatin and protamine at pH 7.0 because coacervate drops are formed under these conditions. To chec, this hypothesis, the activity of α-amylase was determined in a mixture consisting of the same solutions and in the same proportions as those used in the study of the activity of α-amylase in coacervates, but at pH 5.2-5.5, and also in a solution containing starch alone.

The chart showed that the retardation of the reaction is due to a specific action of the protamine. A similar property of alkaline protein was also noted by Liebl, who observed a decrease in the rate of digestion of serum albumin with papain in the presence of the protamine clupein. As we know, clupein also belongs to the protamine group [211].

However, the nature of the action of α-amylase remains unchanged. In both solutions and coacervates, the enzyme hydrolyzes starch dextrins.

The hydrolysis of starch to maltose was modeled by means of β-amylase [148], which cleaves polysaccharides with the participation of water mainly to maltose. In this case, the enzyme used was β-amylase (β-1,4-glucan; maltohydrolase 3.2.1.2), isolated from soybeans by using a slightly modified method of Tauber [656, 686, 828, 927]. The preparation was soluble in water and had a high saccharifying capacity in the 3.6-7.0 pH range. Therefore, in order to study its action, protein-carbohydrate coacervates forming at pH 4.4-4.8 were taken. /152

In order to obtain the coacervate, from 0.25 to 1.0 ml of a 1% soluble starch solution and 0.5 ml of a 0.05% β-amylase solution were added to 2 ml of a mixture of 0.67% solutions of gelatin and gum arabic (in the ratio of 5:3). All the solutions were first formed. A test with iodine showed that starch was present in both the drops heated to 42°. The mixture was then acidified to pH 4.92-4.85, so that coacervate drops and the equilibrium liquid. The starch content was found after it was hydrolyzed with 2% HCl to glucose, which was then determined by the micro metnod Bjerri [32].

The distribution of starch between the drops and the equilibrium liquid as a function of its amount in the total coacervate is shown in Table 35.

The data of Table 35 shows that the fraction of coacervate drops contains 2-4 times

Table 35

DISTRIBUTION OF STARCH IN COACERVATE

Experiment No.	Drops (in mg)	Equilibrium liquid (in mg)	Drops (in mg/ml)	Equilibrium liquid (in mg/ml)	Drops Coacervate	Drops Equilibrium liquid
1	0.178	2.02	1.78	0.70	2.00	2.50
2	0.334	2.2	3.34	0.76	4.00	4.40
3	0.371	10.42	3.70	3.00	1.00	1.20

*In experiments 1-2, the volume of the drops was 0.1 ml, of the equilibrium liquid 2.9 ml, and of the entire coacervate 3 ml. In experiment 3, volume of drop 0.1 ml, equilibrium liquid 3.9 ml; total coacervate 4 ml.

as much starch as the total coacervate; hence, the starch concentrates in the drops.

The absolute values of the amount of soluble starch in coacervate drops at a total starch content of 2.55 mg and 10.79 mg are very similar and respectively equal to 0.334 mg and 0.371 mg, obviously indicating a definite limit of saturation of the coacervate drops with starch.

The ratio of the starch of the drops to the starch of the equilibrium liquid is most favorable when the system contains 2.55 mg of starch.

This was the value taken for studying the action of the enzyme in the coacervate.

The activity of β-amylase was measured after incubating the entire coacervate for 15 min. The reducing sugars (chiefly maltose), formed in the hydrolysis of starch by the enzyme, were measured in milliliters of $KMnO_4$ [148]. /153

The fraction of coacervate drops amounted to 5.05 ml, and the fraction of the equilibrium liquid, to 1.15 ml; the drop coacervate ratio was 4.2, and the drop: equilibrium liquid ratio, 4.4.

The action of β-amylase in the entire coacervate is shown in Fig. 56, form which it follows that the enzyme hydrolyzes the starch in both the drops and the equilibrium in the equilibrium liquid. This shows that the starch in the drops breaks down at a higher rate than in the equilibrium liquid.

Specially designed experiments showed that the maltose found in the drops is formed by the action of the enzyme on the starch.

Thus, hydrolysis of starch by α- and β-amylase can proceed not only in homogeneous solutions but also in coacervate drops.

In these studies, each enzyme cleaves the starch in a coacervate of a single given composition.

However, the same enzyme can be active in coacervates obtained from different

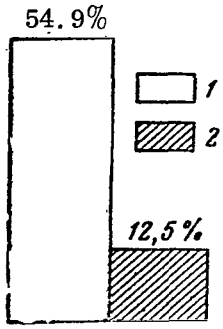


Figure 56. Cleavage of Starch with the Enzyme β-amylase in Coacervate (in % of total amount based on 1 ml). 1-drops; 2-equilibrium liquid

compounds, For example, in studying the effect of ribonuclease on RNA, use was made of different coacervates [251, 262, 267, 325, 327]. /154

Ribonuclease cleaves RNA and other polynucleotides in accordance with the equation

$$(X—R—P)n + H_2O \xrightarrow{\text{ribonuclease}} (X—R—P)n—m + (X—R—PO_4),$$

where X is a purine or pyrimidine base; R is ribose; P is a phosphoric acid radical; X-R-PO_4 is a mononucleotide.

Ribonuclease (polyribonucleotide-2-oligonucleotidotido-transferase cyclyzing 2.7.16) was isolated from calf pancreas [176, 434, 911].

The enzyme was active in the range of pH 4.0-9.5. Its effect on RNA was studied in different coacervates: in protein-carbohydrate, protein-nucleic and lipoprotein coacervates [329-331].

The protein-carbohydrate coacervate in which the behavior of ribonuclease was very thoroughly investigated was obtained as follows.

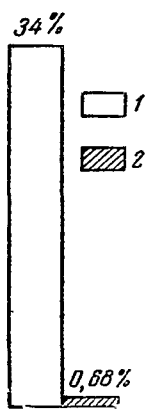


Figure 57. Cleavage of Ribonucleic Acid by the Enzyme Ribonuclease in a Nucleic-Protein-Carbohydrate Coacervate (in % of total amount) 1-drops; 2-equilibrium liquid

To 0.9 ml of a 1% serum albumin solution were added 0.1 ml of a 0.005% ribonuclease solution, 0.9 ml of a 1% gum arabic solution and 0.1 ml of a 1% RNA solution. The serum albumin, RNA and gum arabic were prepared with 0.1 M acetate buffer of pH 4.1; the final pH of the entire coacervate was 4.1, and the temperature was 37°. The activity of the enzyme was determined by measuring the nucleic phosphorus per 1 mg of RNA.

The distribution of RNA and action of the enzyme in the protein-carbohydrate coacervate are shown in Tables 36 and 37 and Fig. 57. /155

RNA concentrates primarily in the drops, where the concentrate increases as by a factr of approximately 42 as compared to the initial value in the entire coacervate. The activity of the enzyme is 50 times as high in the drops as in the equilibrium liquid. In the drops, the amount of RNA decreases by 34% in one hour as compared to the start of the incubation.

The nucleotides formed as a result of hydrolysis of nucleic acid are not held by the drop but pass into the equilibrium liquid [129]. The content of nucleotides in the equilibrium liquid increases. Thus, the increase in the "nucleic phosphorus" in the equilibrium liquid is due to the addition of nucleotides. Conversely, a decrease of "nucleic Phosphorus" in the drops results from hydrolysis of RNA and the discharge of the nucleotides from the drops.

Table 36

DISTRIBUTION OF RNA
IN PROTEIN-CARBOHYDRATE COACERVATE

System	RNA content		Drops	Drops
	mg	mg/ml	Coacervate	Equilibrium liquid
Drops	6.04	30.20	42	274
Equilibrium liquid	1.07	0.11	—	—

Table 37

ACTION OF RIBONUCLEASE ON RNA
IN PROTEIN-CARBOHYDRATE COACERVATE

Time, min	Nucleic phosphorus based on 1 mg of RNA·10-8		Time, min	Nucleic phosphorus based on 1 mg of RNA·10-5	
	Drops	Equilibrium liquid		Drops	Equilibrium liquid
0	934	495	45	623	863
15	754	758	60	611	908
30	672	834			

In this system, serum albumin and gum arabic can form coacervate drops in the absence of both RNA and ribonuclease. Despite the fact that RNA and ribonuclease are additional components, they also participate in the building of the drop. This was successfully demonstrated by studying drops under an electron microscope [273, 367]. /156

Figure 58 shows the form of the drops before and after the action of ribonuclease. A comparison of the pictures clearly shows that cleavage by the enzyme of RNA present in the drop leads to a change of structure: channels appear in the drop, and the latter rapidly disintegrates.

The disintegration of the drops as a result of the action of ribonuclease was also observed in another protein-nucleic coacervate. In this system, nucleic acid was not only the substrate but also a necessary integral part of the drop [327].

In order to obtain such a coacervate, to 0.5 ml of a 1% histone solution were added 0.5 ml of a 10% RNA solution, 1 ml of a 1% histone solution and 0.25 ml of a 0.002% ribonuclease solution. The histone and RNA solution were prepared from 0.2% borate buffer with pH 9.5; the pH of the entire coacervate was 9.5. The activity of the enzyme was determined from both the change in the amount of cleaved RNA and the accumulation of free nucleotides.

Tables 38 and 39 show the distribution of RNA between the drops and the equilibrium liquid and also the action of the enzyme in the coacervate. /157

It is apparent from the data of Tables 38 and 39 that RNA and the enzyme concentrate in the drops of the protein-nucleic coacervate. The activity of the ribonuclease of

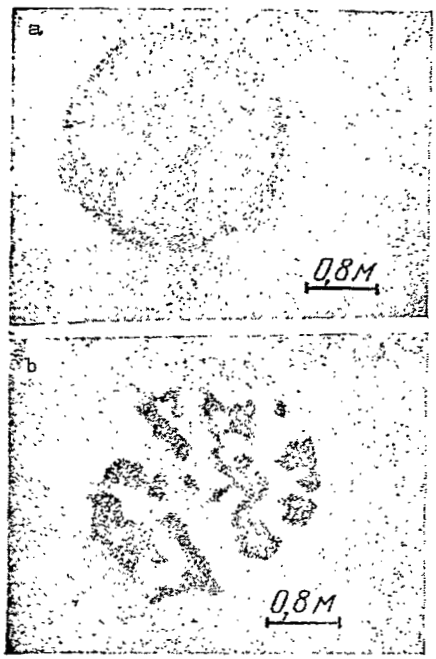


Figure 58. Action of the Enzyme Ribonuclease in Ribonucleic Protein Coacervate. Appearance of coacervate drops under electron microscope (after A.I. Oparin, I.G. Stoyanova and K.B. Serebrovskaya)
a-prior to the action of ribonuclease; b-after the action of ribonuclease

the drops is 20 times as high as that of the enzyme in the equilibrium liquid. After 15 min, about 10% of the total RNA of the drops is hydrolyzed to nucleotides by the ribonuclease. Such a picture is characteristic of the coacervate into which no additional RNA was introduced. If 5 mg of RNA is additionally introduced into the coacervate, the direction of the reaction changes sharply. In the same 15 min, the amount of nucleotides increased by a factor of 2, and the RNA of the drops remained unchanged.

It is well-known that the action of ribonuclease is inhibited by histone [832], and therefore such a model is interesting in that the enzyme, owing to its low activity, acted relatively slowly on the RNA of the drops. The arrival of new portions of RNA from the equilibirum liquid prevented the drops from disintegrating. Two explanations are possible: either the ribonuclease hydrolyzed the RNA which formed a drop together with histone, and the additional RNA replaced it, or the excess RNA, present in the free state in the drops, was replenished at the expense of the RNA of the equilibrium liquid and was hydrolyzed by the enzyme. In either case, the reaction was continuous, the nucleotides left the drops, the disintegrated RNA was replaced by new RNA molecules, and the process was repeated. Thus, the drop assumed the characteristics of steady-state systems.

The overall process may be represented as follows:

$$\text{RNA} \quad \text{RNA} \xrightarrow{\text{Ribonuclease}} \text{Nucleotide}$$

Interesting data along these lines were obtained for a coacervate consisting of RNA and ribonuclease [287], in which both the substrate and enzyme were the necessary components in the drop. In this case, the enzyme played the part of not only the catalyst but also the protein of the drop.

The method of preparation of the coacervate consisted in the following:

A 1% solution of RNA in 0.1 M acetate buffer was combined with a 0.1% aqueous solution of ribonuclease at various pH's, 4.5-5.5. The suspension of nucleoproteins formed was separated by centrifuging and washed off from the excess of these compounds. The precipitate was then suspended in a buffer solution, and globular drops were formed. The average diameter of the drops was 1 mμ; in addition, larger and finer drops were also present in the system. Photographs of such drops under the electron microscope are shown in the original study [287].

The activity of the ribonuclease was determined from the degree of turbidity of

Table 38

DISTRIBUTION OF RNA
IN PROTEIN-NUCLEIC COACERVATE

System	RNA content		Drops	Drops
	mg	mg/ml	Coacervate	Equilibrium liquid
Drops	3.38	67.6		
Equilibrium liquid .	1.63	0.44	54	154

Table 39

ACTION OF RIBONUCLEASE IN DROPS
OF PROTEIN-NUCLEIC COACERVATE
(based on 1 mg of RNA)

Time, min	RNA	Nucleotides discharged from drops	5 mg of RNA added	
			RNA	Nucleotides discharged from drops
0	3,380	—	3,540	—
5	3,310	0,070	3,740	0,266
10	3,070	0,300	3,760	0,415
15	3,050	0,330	3,740	0,630

the solution. The turbidity was proportional to the quantity of the drops. During the enzyme reaction, the drops disintegrate, and the turbidity decreases.

The following indices were obtained for a droplet 1 mμ in diameter: volume, $5 \cdot 10^{-13}$ cm^3, weight $6.2 \cdot 10^{-13}$ g, 87% of the drop consisting of ribonuclease. The /158
ribonuclease in the drop was apparently surrounded by nucleic acid. However, there is no direct evidence of such distribution of RNA. In one hour, $5.4 \cdot 10^{-13}$ g of the ribonuclease of the drops could hydrolyze $2.5 \cdot 10^{-11}$ g of RNA, i.e., almost 100 time as much as the weight of the enzyme. The diffusional influx of RNA from the equilibrium liquid surrounding the drop could give $8.2 \cdot 10^{-6}$ g of RNA. This amount exceeded the possibilities of the drops in one hour. Hence, a saturated layer of the substrate RNA was formed around the drops. The presence of such a layer around an enzyme is of essential importance for the general theory of enzymic action [287, 355]. This layer is gradually hydrolyzed by the enzyme of the drop; the droplet continued its effect for 14 hours, i.e., was in a steady and stable state. When a depletion of RNA began in the equilibrium liquid, the influx of RNA into the drop ceased. The ribonuclease began to cleave the RNA of the external layer of its own drop, and the latter disintegrated.

Thus, being an integral part of the drops or concentrating in the drops obtained from other componds, ribonuclease performs the hydrolysis of RNA. The chemical nature of the drops has a certain influence on its action. For example, histone decreases the activity of the enzyme. Consequently, the presence of histone may change the activity of ribonuclease. In ribosomes, which consist of a protein similar in properties

to histone and RNA, ribonuclease was found in many cases, but the enzyme did not disintegrate the RNA of the ribosome.

The regulation of the activity of ribonuclease in a cell also depends on the lipoproteins of the membranes [118]. The arrangement of the molecules of lipoproteins in the cellular membranes is very similar to that in the envelopes of lipoprotein coacervare drops.

The behavior of ribonuclease was studied in a lipoprotein coacervate of the following composition [331].

To 2 ml of a K oleate solution (3.7 mg) prepared with borate buffer of pH 9.4 (7 mg), 0.5 ml of ribonuclease (0.001 mg) and 1 ml of RNA solution (6 mg) were added.

The activity of the enzyme was measured from the amount of nucleotides formed following the hydrolysis of RNA after 15 min of incubation at 37°.

During this period, 20% of RNA in the coacervate and 53% in the buffer solution was decomposed.

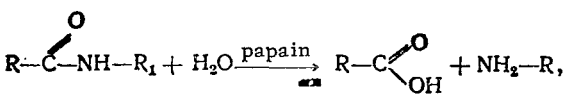
Thus, potassium oleate decreases the activity of ribonuclease in the coacervate by 37%. The inhibiting influence of potassium oleate is regarded as being the result of formation of a saltlike bond between the positive groups in the enzyme molecule and the negative ions of oleic acid present on the surface of an oleate micelle [331]. /159

A gradual addition of KCl to the coacervate decreases the inhibiting action of oleate and under optimum conditions, and the activity of the enzyme is completely restored. In the authors' view, KCl depresses the dissociation of the ions of K oleate. Thus, the oleate cannot bind the positive groups of the enzyme on the surface of the oleate micelle, and the activity of ribonuclease is restored. In oleate coacervates, proteins also affect the activity of ribonuclease [330].

In the systems considered thus far, ribonuclease was the only enzyme. However, it was found capable of hydrolyzing RNA in a complex protein-carbohydrate coacervate system which also contained another enzyme, papain.

This coacervate was used to model the process of decomposition of a protein where the major part was played by papain [211].

Papain (3.4.4.10) was isolated from the juice of papaya Carica papaja. The enzyme thus hydrolyzes proteins which are in an isoelectric state, in accordance with the equation [774]:



where R and R_1 are radicals of the side groups of the polypeptide chain.

In order to study the action of papain, a protein-carbohydrate coacervate with the following composition was prepared [217].

To 5 ml of a 0.25% solution of Na arabinate was added 5 ml of 0.25% serum albumin. The mixture was brought to pH 3.5 by acidifying with 0.1 N HCl, so that drops were formed. Papain, which is well sorbed (absorbed) by the drops, was then added.

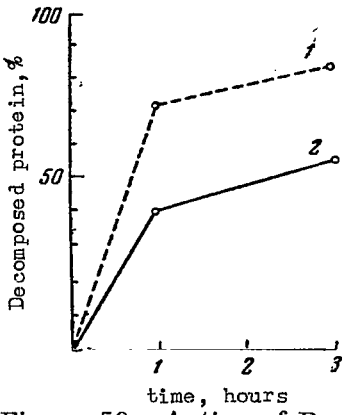


Figure 59. Action of Papain on Serum Albumin. 1-serum albumin-sodium arabinate; 2-serum albumin

The substrate — serum ablumin — and the enzyme were concentrated in the drops. The proteolysis was studied at 38°. In addition, the effect of RNA, AMP, ribonuclease, histone and Na_2SO_4 on this process was studied. Each of these compounds was added to the coacervate. After the removal of uncleaved serum albumin, the amount of protein hydrolyzed by the enzyme was determined by the method of Folin and Anson. /160

It is apparent from Fig. 59 that the most complete hydrolysis of the protein took place in the coacervate of serum albumin and Na arabinate.

AMP, histone, Na_2SO_4 and particularly RNA inhibit this reaction. Upon addition of ribonuclease, RNA was frequently cleaved by the enzyme, and a certain increase of the protoelysis was then observed.

Thus, ribonuclease like papain, α- and β-amylases, being included in coacervate systems, was found capable of splitting natural polymers (protein, RNA, starch) to low molecular products.

Synthetic Reactions

A synthesis of starch and polyadenylic acid from low molecular compounds was carried out in coacervates by using the enzymes phosphorylases [149-151, 265, 266].

Potato phosphorylase [α-1, 4-glucan: orthophosphate glucosyl transferase (2.4.1.1.)] [176] was taken as the enzyme synthesizing starch.

This enzyme synthesizes and cleaves a polysaccharide in accordance with the equation

$$n \cdot C_6H_{11}O_5—O—PO_3H_2 \rightleftarrows (C_6H_{10}O_5)\,n + n\,H_3PO_4.$$

Depending on the pH and the proportion of the components, the equilibrium of the reaction may be shifted toward the synthesis of breakdown of polysaccharides [230, 695].

It should be emphasized that phosphorylase is unusually sensitive to the reaction of the medium. Its activity is limited to the relatively narrow pH range of 5.8-7.2 [495].

The phosphorylase preparation, isolated from Lorch grade potato by a special composite technique [129, 808, 957], had an optimum of synthesizing activity at pH 6.0-6.2. The enzyme was included in a protein-carbohydrate coacervate stable at this pH.

In order to obtain the coacervate, 0.1 ml of a 0.1% solution of soluble starch was combined with 0.1 ml of NaF (0.69 mg), 0.3 ml of 0.5 M acetate buffer (pH 6.0-6.2), 0.5 ml of a 0.67% gum solution, 0.5 ml of an enzyme solution of various concentrations, 0.15-0.30 ml of a glucose-1-phosophate solution (3.75 mg), and 0.4 ml of a 1% histone solution. The latter was isolated from the thymus gland by Hnilica's method [719].

In such a coacervate, the substrate for the synthesis of starch was dipotassium salt of glucose-1-phosphate, which was uniformly distributed between the drops and the equilibrium liquid.

As follows from the reaction equation, the synthesis of starch from glucose-1-phosphate forms a polysaccharide, and orthophosphoric acid is liberated. Therefore, /161
the rate of the synthesis can be evaluated by measuring both the polysaccharide and the inorganic phosphorus of orthophosphoric acid [16, 37, 387, 710, 796]. It was found impossible to determine the synthesis of starch in coacervates from the increase of inorganic phosphorus, since the latter was bound by histone during the removal of impurities from the solution. A specially designed model experiment with addition of $Na_2HPO_4 \cdot 2HO$) gave the same results. Obviously, histone forms a stable complex with phosphate. The rate of synthesis was therefore determined from the accumulation of the polysaccharide.

It is known that potato phosphorylase from which impurities have been thoroughly removed synthesizes po.ysaccharides, chiefly polysaccharides with straight chains. Branching of the chain depends on the associated Q-enzyme which at the present time is designated as the branching enzyme (α-1-4-glucan: 1,4-glucan, 6-glucosyl transferase 2.4.1.18) [176, 491, 710, 867]. In addition to the phosphorylase, the prepara- /162
tion used in the coacervate contained the branching enzyme.

Table 40

SYNTHESIS OF STARCH IN COACERVATES AT DIFFERENT ENZYME CONCENTRATIONS

Color	Dilution of enzyme						
	1	1/2	1/5	1/10	1/20	1/40	1/80
	Rate of appearance of color, min						
Gray	none	none	3	5	10	20	60
Lilac	3	3	20	20	60	60	none
Violet	10	10	60	60	none	none	»
Blue	60	90	none	none	»	»	»
Dark blue	90	none	»	»	»	»	»

The structure of the synthesized polysaccharide depends on the structure of the polysaccharide taken as the seed [196, 197, 491] and used as the matrix. The role of the seed was played by soluble potato starch, whose amount was so slight that it was not stained by iodine in the coacervate.

The rate of synthesis was determined from the staining of the starch formed by the solution of iodine in potassium iodide by two methods.

1. From the color changes, beginning with a colorless gray, then lilac and bluish-violet in samples taken at definite time intervals.

2. By measuring the density of the blue color after a given time segment at 580 mμ in a photoelectrocolorimeter [769, 843, 844].

Table 41

SYNTHESIS OF STARCH IN COACERVATES

Fraction	Time of synthesis min	Starch, mg/ml	Drops Equilib. liquid	Synthesis* %
Drops	30	3.3—3.4	22—24	20.24—20.85
Equilibrium liquid .		0.14—0.15		0.92
Drops	120	5.0	22	30.79
Equilibrium liquid .		0.23		1.39
Glucose-1-phosphate added after the formation of drops				
Drops	30	2.9	22	17.70
Equilibrium liquid .		0.13		0.80

*The % synthesis of starch in the entire equilibrium liquid and in the fraction of coacervate drops was calculated with respect to starch (taken as 100%) which could have been formed from the total glucose-1-phosphate added to the coacervate.

In this case, the coacervate drops were dissolved in 0.5 M acetate buffer. The corresponding amount of buffer was also added to the equilibrium liquid.

The results of the measurements are shown in Tables 40 and 41 and in Fig. 60.

It follows from the data of Tables 40 and 41 that as the enzyme concentration decreases, the initial stages of the synthesis and also the entire reaction slow down.

At the same concentration of enzyme, and as the synthesis time increased from 30 to 120 min, the starch content of the drops and of the equilibrium liquid increased by a factor of approximately 1.5.

During the same amount of time, 22 times as much starch accumulates in the coacervate drops as in the equilibrium liquid. Special experiments showed that the enzyme concentrates mainly in the drops and synthesizes therein a starch from glucose-1-phosphate, which freely penetrates from the equilibrium liquid into the drops, since the order of addition of glucose-1-phosphate before or after the formation of the drops has an insignificant effect on the rate of the synthesis.

The starch content surpasses several fold the possible amount which could have been synthesized in the drops in accordance with the initial distribution of glucose-1-phosphate in the coacervate without the enzyme; in all, only 0.10 mg of this compound should be present in the drops. Consequently, glucose-1-phosphate must have entered the drops from the equilibrium liquid as the starch was gradually synthesized in them. Obviously, the synthesis of starch must be associated with an increase in the weight of the individual drops [149-161, 673]. /163

Synthesis of Starch in Individual Drops. The method of interference microscopy was successfully used to measure the increase of starch in individual drops. At first, drops containing starch were suspended, independently of the time of synthesis, and they were then suspended for different durations of this reaction. Table 42 demonstrates

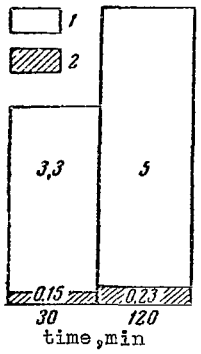


Figure 60. Synthesis of Starch in a Multicomponent Coacervate (in mg/ml) 1-drops; 2-equilibrium liquid

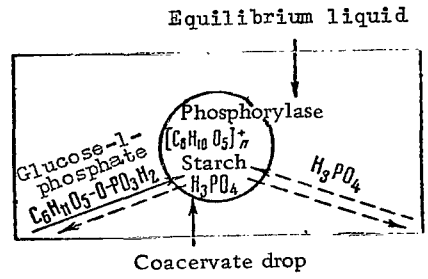


Figure 61. Diagram of Enzymatic Synthesis of Starch in a Coacervate Drop.

changes in the volume, weight and concentration of substances which took place in the drops during the synthesis of starch [136].

As can be seen from these data, the size and dry weight of the drops increase. In blank determinations with the inactivated enzyme (in the absence of synthesis), the size and weight of the drops did not change. In the entire coacervate system during the synthesis of starch, the total weight of histone + + gum arabic + glucose-1-phosphate and other components remained constant. All the compounds from which the drops were built up and also the enzyme were concentrated mainly in the drops, with the exception of glucose-1-phosphate. For this reason, the increase in the dry weight of the mass of the drop was due to the synthesis of starch therein. For example, for drop No. 1, the weight increase is $5.8 \cdot 10^{-12}$ g, which amounts to 50.7% of the original weight of the drop and 33.7% relative to the weight of the drop after 45 min. The total concentration of substances per unit volume decreased by approximately 2-3%. Thus, a fine droplet was found to be denser than a large one. The same phenomenon was also observed in numerous drops of the same coacervate and also in coacervate drops differing in chemical composition.

The synthesis of starch in a coacervate drop is shown as a diagram in Fig. 61. During the reaction, there take place a consumption of glucose-1-phosphate and its transformation into starch, which deposits in the drop and escapes from the sphere of the reaction. The equilibrium is disturbed, and fresh amounts of glucose-1-phosphate are required for its reestablishment. /164

Thus, the synthesis of starch proceeds at the expense of glucose-1-phosphate present in the drop in the initial stage of the synthesis and also coming from the outside during the succeeding periods of this reaction (in the drawing, the solid arrow indicates the basic movement of glucose-1-phosphate during the synthesis of starch in the drop, and the dashed arrow indicates the direction of the possible escape of glucose-1-phosphate from the drop upto the start of synthesis of starch therein). Since the composition of the coacervate drop included histone, in the presence of which no free orthophosphoric acid could be determined, the possible movement of H_3PO_4 is denoted by dashed lines.

Starch synthesized by phosphorylase in the drops was cleared to maltose in the presence of α-amylase. In this case, the equilibrium liquid without starch but with with α-amylase was added to the drops with starch. Such a coacervate was incubated

Table 42

SYNTHESIS OF STARCH IN INDIVIDUAL COACERVATE DROPS

Drop No.	Time, min	Diameter 10^{-4} cm	Volume 10^{-11} cm^3	Weight 10^{-12} g	Concentration, %
1	10	3,74	27.4	11.1	41
	30	4.40	44.5	17.0	38
	45	4.40	44.5	17.3	39
2	10	5.88	106.2	34.0	32
	25	6.93	173.9	48.7	28
	40	7.77	245.1	76.2	27
3	10	6 3	130.6	37.9	29
	25	7.77	245 1	58 3	24
	40	8.19	287,0	60.3	21
4	10	2.1	4.84	2.4	50
	20	2.52	8.36	3.9	47
5	10	2.52	8.36	4.5	54
	15	2.94	13.28	6.8	51
6	10	3.36	19.82	7.1	36
	30	6.09	118.01	24.8	21
7	10	4.2	38.71	9.7	25
	15	5.67	95.24	22.8	24
8	10	4.62	51.52	14.4	28
	30	6.51	140.21	29.4	21
9	10	4.2	38.71	12.4	32
	20	5.04	66.89	17.4	26
10	10	5.25	75.61	21.9	29
	20	5.88	106.22	26.5	25

for an hour at 18°, and then maltose was determined in the drops and in the equilibrium liquid by means of paper chromatography [355].

Thus, a stream of substances can be produced by the combined action of two enzymes (phosphorylase and α-amylase).

A model of the stream consists in the fact that glucose-1-phosphate penetrates into the coacervate drop from the surrounding medium as the initial compound, and the equilibrium liquid is penetrated by maltose, which was earlier absent from it [129].

We should emphasize the major part played by the synthesized polysaccharide as polymer, which, being the substrate for α-amylase, did not disturb the overall course of the reaction, but promoted it.

Starch was the first polymer whose synthesis was successfully observed in a coacervate drop.

The second polymer was polyadenylic acid, synthesized in a coacervate system by means of polynucleotide phosphorylase (polynucleotide - nucleotidyl transferase 2.7.16) [107-108, 263, 266, 268, 326]. The enzyme performs the synthesis and break-

down of polyadenylic acid according to the equation

$$n\cdot\text{ADP} \underset{\longleftarrow}{\xrightarrow{\text{Enzyme}}} [\text{AMP}]_n + n\cdot H_3PO_4.$$

The direction of the reaction toward this synthesis or breakdown depends on the proportion of the components, chiefly on the amount of inorganic phosphate. Usually, in the presence of excess H_3PO_4, the cleavage of the polynucleotide begins [107-108, 833]. The source of the enzyme was the microorganism Micrococcus lysodeikticus. The preparation of polynucleotide phosphorylase had a good activity at pH 9.5 and was included in the nucleotide coacervate containing mineral salts.

The coacervate was formed by combining equal volumes of solutions I and II. Solution I included 0.5 ml of 0.0078 M $MgCl_2$, 0.5 ml of 1.2 M KCl, and 0.4 ml RNA (26 mg) prepared from 0.12 M tris-buffer.

Solution II consisted of 0.5 ml of histone (26 mg of protein), and 0.5 ml of polynucleotide phosphorylase containing 2 mg of protein.

ADP, which served as the substrate for the synthesis of polyadenylic acid, was added to the ready coacervate. Like other mononucleotides, ADP readily penetrates into the drops. Usually, ADP distributes itself more or less uniformly between the drops and the equilibrium liquid [129].

The activity of the enzyme was measured from the phosphorus of ADP and polyadenylic acid.

It was first established that RNA and histone, which enter into the coacervate, do not depress the enzyme.

The action of the enzyme in the coacervate was measured as a function of the incubation time at 37° (Tables 43 and 44).

It is apparent from these data that polynucleotide phosphorylase synthesizes polyadenylic acid in the drops from the ADP entering into them from the equilibrium liquid.

When the ions of inorganic phosphorus are tied up, the reactions will proceed up

Table 43

SYNTHESIS OF POLYADENYLIC ACID IN THE ENTIRE COACERVATE
(in μ-moles of P)

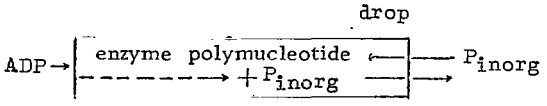
Time, min	ADP	Polyadenylic acid + RNA
0	11,930	0,162
180	11.220	0,998
Changes	—0,710	+0.836

Table 44

SYNTHESIS OF POLYADENYLIC ACID IN COACERVATE
(in μ-moles of P)

Incubation time, min	Drops (polyadenylic acid + RNA)	Equilibrium liquid (ADP)
0	4.55	13.000
180	5.45	11.900
Changes	+0.90	—1.100

to a complete conversion of ADP into polyadenylic acid, which deposits in the drops as a polymer and is removed from the reaction. The latter proceeds as follows:



Owing to the fact that the enxyme concentrates in the drops, the activity of polynucleotide phosphorylase in the latter was found to be one-fourth of that in the equilibrium liquid and amounted to 80% of the total activity of the enzyme in the entire coacervate.

In this system, the synthesis of polyadenylic acid proceeded in the ready drops consisting of histone + RNA. Polyadenylic acid itself can also form coacervate drops in the presence of proteins.

The preparation of a complex protein - polynucleotide coacervate in the process of enzymatic synthesis of polyadenylic acid was also carried out in the following mixture: 6.3 mμ modes of ADP, 3.9 mμ moles of $MgCl_2$, 600 mμ moles of KCl, 60 mμ moles of tris-buffer pH 9.5, 1.19 mg of polynucleotide phosphorylase and 15 mg of histone The total volume was 3 ml, and the final pH of the solution, 9.5. The mixture was incubated at 37°. The ADP present and histone did not form any coacervates. Therefore, at the start of the incubation the mixture was an ordinary transparent solution [263, 326]. /168

However, as the synthesis of polyadenylic acid from ADP under the influence of the enzyme proceeded further, the picture changed. Polyadenylic acid combined with histone, and coacervate drops were formed.

Figure 62 shows the process of synthesis of polyadenylic acid in the presence and absence of histone.

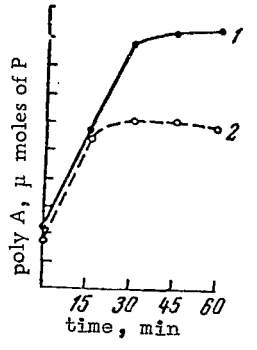


Figure 62. Synthesis of Polyadenylic Acid by the Enzyme Polynucleotide Phosphorylase
1-in the presence of histone; 2-in the absence of histone

It follows from the data of Fig. 62 that in the presence of histone the process of synthesis is considerably accelerated. At the same time, owing to their large molecular weight, the polymers in effect lead the reaction and thereby promote the reaction in the direction of their synthesis. Naturally, the formation of new complexes also speeds up the synthesis. However, the main process appears to be the formation of drops in which the enzyme concentrates.

Oxidation-Reduction Reactions

The synthesis of natural polymers proceeds virtually without any additional consumption of energy. The energy is stored in the substrates in the form of energy-rich phosphorus bonds. A major part in the formation of these bonds is played by one of the universal energy-transfer agents, ATP [308, 777, 893]. The synthesis of ATP in organisms proceeds along two paths, the photosynthetic and oxidative paths. Photosynthetic phosphorylation is accomplished by means of the energy of solar rays, and exidative phosphorylation by means of the oxidation of organic molecules during oxidation-reduction processes, for instance during respiration [702].

In the respiration chain, there are several stages where the formation of ATP takes place [98, 104-106, 353, 354, 724, 893], and first of all during the transfer of hydrogen from NAD to FAD. NAD can be reduced to NAD-H_2 directly via removal of H_2 from the compound being oxidized. Reactions of this kind are thought to have been of major importance during the initial periods of the existence of the earth, when oxygen was absent from the atmosphere. At the present time, in any organism, the process of removal of hydrogen from organic compounds involves the participation of NAD and corresponding dehydrogenases. A modeling of the oxidation-reduction reaction involving /169
a dehydrogenase was carried out in a protein coacervate [276, 325]. The enzyme employed was a dehydrogenase isolated from cytoplasmic membrances of Micrococcus lysodeikticus. The enzyme had the ability to dehydrogenate malic acid and also to remove hydrogen from NAD-H_2 and transfer it to dyes, for example 2, 6-dichlorophenol-indophenol (DCPI), in accordance with the equation

$$\underset{\text{(bluish-green)}}{\text{NAD} - H_2 + \text{DCPI}} \xrightarrow{\text{enzyme}} \underset{\text{(colorless)}}{\text{NAD} + \text{DCPI} - H_2}$$

The dehydrogenase was included in the protein coacervate at pH 7.4.

Composition of coacervate: 1 ml of 1% serum albumin solution, 0.1 ml of enzyme containing $280 \cdot 10^{-6}$ g of protein, 1 ml of 1% histone solution, 0.8 ml of 0.1 M solution of phosphate buffer of pH 7.4. The same buffer was used to prepare solutions of histone and serum albumin. In addition to these compounds, the mixture contained 0.5 ml of NAD-H_2 and 0.4 ml of the dye solution.

The activity of the enzyme was measured from the oxidation of NAD-H_2 in NAD and from the rate of decolorization of the dye.

The coacervate proteins, serum albumin and histone depressed the ability of the enzyme to hydrogenate malic acid and did not affect its activity with respect to NAD-H_2.

NAD-H_2 was evenly distributed between the drops and the equilibrium liquid, and the dye was present primarily in the drops.

Figure 63 shows the process of dehydrogenation of NAD-H_2 in the entire coacervate and in the equilibrium liquid as a function of the reaction time [325].

Practically all of the enzyme was concentrated in the drops. If the reaction rate in the drops is compared to the reaction rate in the buffer solution, and the unit of rate taken is the amount of DCPI decolorized in the first minute of the reaction and is expressed in percent of the total DCPI content, the ratio of the reaction rate in the drops to that in the solution will be 58.

The concentration of enzyme calculated per unit volume in the drops was 133 times as high as in the solution. As follows from the above, in the drops, the dehydrogenation process went 58 times as fast as in the buffer solution. Hence, the acceleration of the reaction in the drops results from the concentration of the dehydrogenase molecules therein. However, the rate turned out to be less than could have been expected. Evidently, a partial inhibition of the enzyme takes place in the drops. The enzyme in the drops decolorized not only the dye which had already been present in the drops, but also fresh portions of the dye coming in from the equilibrium liquid. Thus, the drop /169

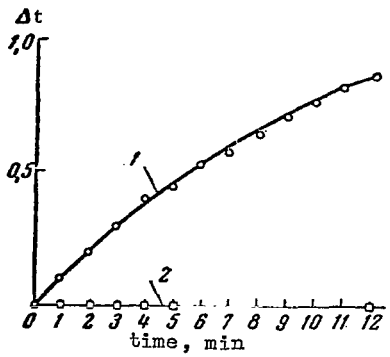


Figure 63. Dehydrogenation of NAD-H_2 by Dehydrogenase in Protein Coacervate
1-coacervate drops; 2-equilibrium liquid

was the site to which NAD-H_2 and the dye flowed from the equilibrium liquid, and by dehydrogenating NAD-H_2, the enzyme reduced the dye.

Like other reactions involving dehydrogenases, this reaction proceeds as a result of the oxidation of already-existing organic compounds, and the energy obtained is not particularly high [919]. Under the reducing conditions prevailing on the earth in the distant past, the predominant reactions were of a type that has endured until the present time, for example, fermentation processes.

One of the products of different types of fermentation is carbonic acid. It is postulated that further evolution went in the direction of the use of CO_2 [309, 922]. Chlorophyll-type fragments [188] assimilated solar energy by converting it for the reduction of CO_2 to organic compounds. According to Calvin [164], such reactions could have taken place during the precellular period in lipoprotein coacervates. In this case, chlorophyll-like pigments acquired a definite orientation which reinforced their ability to photosensitization.

A check of Calvin's point of view was made by Serebrovskaya, Yevstigneyev and others on lipoprotein coacervates containing chlorophyll, in which oxidation-reduction reactions were carried out by using radiant energy [97, 269, 332, 333, 336].

In model experiments with dehydrogenases, the reduction of the dye in the coacervates took place at the expense of NAD-H_2 as the hydrogen donor with the obligatory participation of an enzyme.

In this case, the role of enzyme was played by chlorophyll + light, the source of hydrogen was ascorbic acid, and the acceptor was the dye methyl red.

The reaction proceeded in accordance with the equation:

$$\text{Ascorbic acid} + \underset{\text{(red color)}}{\text{methyl red}} \xrightarrow{\text{chlorophyll}} + h\nu \text{ of light}$$

$$\text{dehydroascorbic acid} + \underset{\text{(colorless)}}{\text{methyl } H_2}$$

/171

Such a reaction was modeled in a lipoprotein coacervate consisting of serum albumin and potassium oleate. The same coacervate was used in a study of the action of ribonuclease.

Two ml of such a coacervate was combined with 1 ml of an alcohol chlorophyll solution of different concentrations at pH 9.5*. The 3-component coacervate, protein -

*Serebrovskaya obtained a new coacervate without ethanol. In this case, chlorophyll reacts with RNA and dissolves in water; the absorption spectrum of the RNA-chlorophyll complex resembles the spectrum of chlorophyll in leaves.

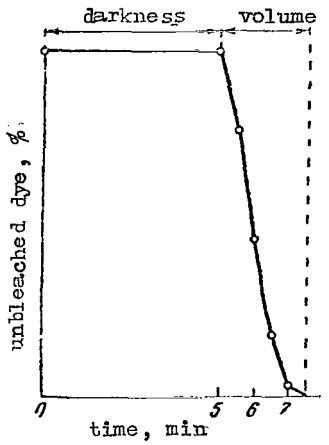


Figure 64. Photosensitizing Action of Chlorophyll

Table 45

REDUCTION OF DYE IN LIPOPROTEIN COACERVATE WITH THE PARTICIPATION OF CHLOROPHYLL AND LIGHT

Amount of chlorophyll (in E_{665})	Rate per unit volume		Drops
	Drops	Equilibrium liquid	Equilibrium liquid
0.24	325	7.75	48
0.120	425	5.25	81
0.060	150	4.25	35

lipid - chlorophyll, was diluted by a factor of 11 with water, and then 40 mg of ascorbic acid and the dye were added to 4 ml of the mixture.

In the coacervate, ascorbic acid was uniformly distributed between the drops and the equilibrium liquid, and chlorophyll concentrated predominantly in the drops. Figure 28d shows a photograph of the drops containing chlorophyll. The coacervate was illuminated at definite time intervals.

The photosensitizing activity of chlorophyll was measured from the amount of decolorized dye, and the content of chlorophyll and dye was measured spectrophotometrically at 665 and 520 mμ respectively (Table 45 and Fig. 64).

The amount of decolorized dye in percent of the total dye content of the coacervate during the first minute of illumination was taken arbitrarily as the unit rate.

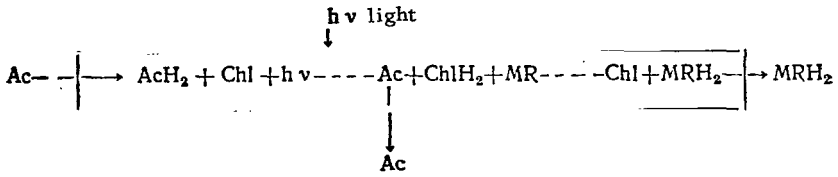
If instead of K oleate, lecithin is taken for the preparation of the coacervate, the reaction is accelerated by a factor of four.

The activity of chlorophyll a and b was studied in detail in such a coacervate, and the influence of various factors on the reaction rate was also investigated.

It was shown that the photosensitizing activity of chlorophyll is considerably increased when the pigment acts in coacervate drops, not in solutions containing individual compounds (components) at which the coacervate is made up [217], 328, 335].

It is quite apparent from the data of Table 36 and Fig. 64 that chlorophyll reduces the dye in the coacervate only after illumination. The reaction does not occur in the dark. Under optimum experimental conditions, the dye is decolorized 81 times as fast in the drops as in the equilibrium liquid. At the same time, the chlorophyll content of the drops is only 54 times as high as in the equilibirium liquid. The disproportionate increase of the reaction rate in the drops can be explained by an ordered pseudocrystalline arrangement of the chlorophyll molecules in the lipoprotein layers of the drops. In chloroplasts, chlorophyll is also located in lipoprotein layers.

The entire process can be represented schematically as follows [325]:



where AcH_2 is ascorbic acid, MR is methylene red, MRH is decolorized methyl red, Ac is dehydroascorbic acid, and Chl, chlorophyll.

According to this equation, the photosensitizing activity of chlorophyll is used for the reduction of the dye. Thus, this confirms Calvin's viewpoint concerning the primary role of chlorophyll-type pigments as sources of reductive reactions using radiant energy.

A further improvement of the action of pigments was directed to their specialization in the reduction of CO_2 to organic compounds with the evolution of free oxygen.

The presence of oxygen presented ample opportunities for oxidation reactions with a high energy yeild. In addition to water, H_2O_2 is also a product of oxidation reactions involving O_2. Hydrogen peroxide is a poison for cells. It is split by special enzymes, of which the most common is catalase.

Coacervates and Catalase. The enzyme catalase is widespread in both the plant and animal worlds [670].

In its chemical nature, catalase is a proteide whose composition includes a prophyrin nucleus with iron and a protein [21, 83, 235, 670, 702, 851].

The catalytic start in the enzyme is iron, which may accumulate in the coacervate drops.

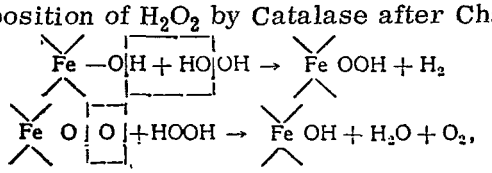
Iron accelerates the breakdown of H_2O_2, but the reaction proceeds slowly. The cleavege of H_2O_2 in artificial models of catalase is somewhat faster [243]. However, the ability of the enzyme itself to decompose H_2O_2 is not comparable to any other agent, since, for example, 1 mg of iron in catalase is equivalent in acitivty to 10 t of inorganic iron. Obviously, the evolution process involved a gradual improvement of iron complexes, which led to the formation of a catalyst as active as catalase.

The basic equation of the reaction of H_2O_2 decomposition by the enzyme is relatively simple:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2.$$

The mechanism of the reaction, proposed by Chance [670], was confirmed in 1963 after a study made by Shonbaum [896].

Decomposition of H_2O_2 by Catalase after Chance



where $\diagup\!\diagdown$Fe-OH is the active group of catalase, firmly bound to the protein

$$HOOH \rightarrow H_2O_2.$$

The catalase of liver and bacteria was taken as the enzyme. The source of bacterial catalase was the lysate of a culture of Micrococcus lysodeikticus containing up to 3-4% catalase. Crystalline bacterial catalase was first isolated from this microorganism [718].

The lysate was supplied to use by Gel'man [257], and the crystalline preparation of catalase from ox liver, by Moiseyenko. The enzymes were included in protein and protein - carbohydrate coacervates at pH 4.2-9.0 [143]. /174

In order to obtain a protein-carbohydrate coacervate of pH 4.27, 0.1 ml of a solution of enzyme of various concentrations, 0.3 ml of a 0.67% gum arabic solution and 0.1 ml of a 0.4% CH_3COOH solution were added to 0.5 ml of a 0.67% gelatin solution. Coacervate drops were formed at 41-43° and pH 4.27.

The protein - carbohydrate coacervate at pH 6.0 was obtained by adding 0.5 ml of a 1.5% protamine sulfate solution to 0.2 ml of a 0.01 M phosphate buffer (pH 6.0), 0.1 ml of a solution of the enzyme of various concentrations and 0.25 ml of a 0.67% gum arabic solution.

Coacervate drops were formed at pH 6.0 and a temperature of 20°.

A protein coacervate of pH 8.8-9.0 was obtained from 0.25 ml of a 1.5% protamine sulfate solution, 0.1 ml of a solution of enzyme of different concentrations, 0.75 ml of a 0.67% of gelatin solution and 0.1 ml of a 0.1% NaOH solution. The drops appeared at /175 pH 8-9.0 and a temperature of 41-43°.

The manganometric method was used for measuring the activity of the catalases [172].

The substrate taken was 0.2 ml of a 0.64% H_2O_2 solution, which was added after the formation of coacervate drops. A certain period of time after the incubation, 0.4 ml of a 10% H_2SO_4 solution was added. Sulfuric acid inactivated the enzyme, and the reaction ceased. The unreacted hydrogen peroxide was titrated with a 0.01 N $KMnO_4$ solution. The activity of the enzyme was expressed in percent of decomposed H_2O_2 of the total amount of H_2O_2 in the blank coacervate where the enzyme was inactivated.

Results of measurement of the activity of bacterial catalase and liver catalase in different coacervates are shown in Tables 46-48. Tables 46 and 47 show data on the cleavage of H_2O_2 in fractions of drops occupying a volume of 0.1 ml and in an equilibrium liquid whose volume was 0.8-1 ml.

Table 48 sums up the action of enzymes expressed in amounts per 1 ml.

As follows from Tables 46-48, bacterial catalase and liver catalase under optimum conditions are completely concentrated in the coacervate drops. The enzyme is almost completely absent from the equilibrium liquid, and the activity of the catalase is very high even at a low concentration of the enzyme ($\sim 1 \cdot 10^{-6}$).

Table 46

ACTIVITY OF BACTERIAL CATALASE IN COACERVATES
(DECOMPOSED H_2O_2 in %)

Fraction	Activity						Experimental conditions
	Protein-carbohydrate coacervate (pH 6.0)						
	Dilution of lysate						
	1:20	1:100	1:150	1:200	1:300	1:400	
Drops	97.93	75.72	59.56	57.86	26.62	21.59	Incubation time 3 min at 41-43°
Equilibrium liquid	1.69	1.46	0.74	2.14	0	0	
	Incubation time						
	2 min		3 min		5 min		
Drops	71.78		76.33		90.32		1:100 dilution of lysate at 41-43°
Equilibrium liquid	4.28		0		3.2		
	Temperature						
	25°		37°		43°		
Drops	37.32		58.96		58.96		1:150 dilution of lysate
Equilibrium liquid	0		0		0		Incubation time 3 min
	Protein coacervate (pH 8.8-9.0)*						
	Dilution of lysate						
	1:100			1:150			
Drops	68.11			57.63			Incubation time 3 min at 41-43°
Equilibrium liquid	13.18			13.56			

*As a result of decomposition of H_2O_2 in the alkaline medium, no more detailed studies were made in the coacervate at pH 8.9-8.0.

Table 47

ACTIVITY OF LIVER CATALASE
AT 41 43° AND A 3 MIN INCUBATION
(decomposed H_2O_2 in %)

Fraction	Enzyme concentration (dilution)		
	1 : 1000	1 : 2000	1 : 10 000
Protein-carbohydrate coacervate (pH 4.27)			
Drops	93.39	74.08	30.12
Equilibrium liquid . . .	1.59	0	0.74
Protein-carbohydrate coacervate (pH 6.0)			
Drops	59.86	—	9.14
Equilibrium liquid . . .	38.82	—	86.14

Table 48

ACTIVITY OF BACTERIAL CATALASE IN COACERVATES
(in % of Decomposed H_2O_2 per unit volume = 1 ml)

Drops	Equilibrium liquid	drops / solution*	drops / equilibrium liquid	Experimental conditions: Variable values	Experimental conditions: Constant values
Catalase of bacteria					
Protein-carbohydrate coacervate (pH 6.0) For different dilutions of lysate					
979,3	1,47	12,2	666,2	1:20	41—43°
757,2	1,27	9,9	596,2	1:100	Incubation 3 min
595,6	0,64	8,1	930,6	1:150	
266,2	0	7,84	∞	1:300	
For different incubation times, min					
717,8	3,73	9,2	192,4	2	Lysate concentration 1:100
763,3	0	9,68	∞	3	41—43°
903,2	2,78	11,29	324,9	5	
At different temperatures, °C					
373,2	0	5,04	∞	25	Lysate concentration 1:150, incubation 3 min
589,6	0	7,7	∞	37	
589,6	0	8,03	∞	43	
Protein Coacervates (pH 8.8-9.0) For different dilutions of lysate					41—43°
681,1	10,98	8,86	62,03	1:100	Incubation 3 min
576,3	11,3	8,5	51,0	1:150	
Liver catalase					
Protein-carbohydrate coacervate (pH 4.27)				Dilution of enzyme	
933,9	1,4	12,0	648,5	1:1000	41—43°
740,8	0	10,2	∞	1:2000	Incubation 3 min
301,2	0,67	9,04	449,5	1:10000	
Protein-carbohydrate coacervate (pH 6.0)					
598,6	33,75	7,5	17,76	1:1000	41—43°
91,4	74,9	1,32	1,22	1:10000	Incubation 3 min

*By solution is meant the activity of the enzyme in 1 ml of buffer solution.

As an example, one can cite bacterial catalase. If the entire volume of the liquid (consisting of the coacervate and H_2O_2 added to it) is taken as unity, then for the bacterial lysate the concentration will be only 1/1300-1/4200, and the lysate will contain approximately 1/100-1/1000 of the enzyme catalase itself. Consequently, upon dilution of the lysate, the enzyme concentration in the entire system is 1/100,000 - 1/1,000,000.

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Figure 65 shows pictures of coacervates in which the process of separation of oxygen bubbles in the form of dark spots has been photographed. In the first two pictures, it was possible to capture the initial stages, and in the two following pictures the decomposition process is already quite advanced. The oxygen bubbles became large (dark circles). The reaction proceeded very rapidly.

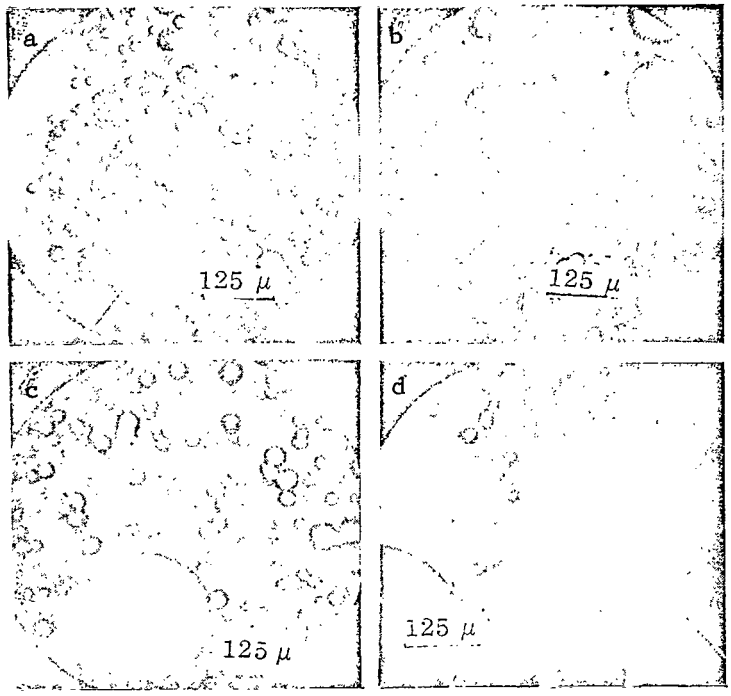

Figure 65. Cleavage of Hydrogen Peroxide with Catalase in Coacervate Drops
a,b-initial stages of reaction; c,d-final stages of reaction; arrows - oxygen bubbles

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The decomposition of H_2O_2 goes practically to completion, since oxygen does not interfere and escapes in the form of bubbles. This process is associated with a change in the size of the drops. A considerable increase in the H_2O_2 concentration of the coacervate and also in the duration of the effectiveness of the enzyme lead to the disintegration of the drops.

It is interesting to note that the catalases behave differently toward the same coacervate.

For example, bacterial catalase is practically completely concentrated in the coacervate of protamine-gum drops at pH 6.0 and splits H_2O_2 to the extent of 97.93%. Under the same conditions, liver catalase becomes uniformly distributed between the drops and the equilibrium liquid, and the decomposition of H_2O_2 in the drops amounts to 59.86%. At the same time, this enzyme concentrates in gelatin - gum drops at pH 4.27 and decomposes H_2O_2 to the extent of 93.9%. / 179

Bacterial catalase is inactive at this pH. Some reactivation of the enzyme was achieved by gradually bringing the pH to 6.0.

Compounds which make up the coacervates — gelatin protamine and gum arabic — when added to buffer solutions containing the enzyme, decrease the activity of catalases to various degrees [129]. When the enzyme is concentrated in the drops under optimum conditions, despite their hard content of protamine, gelatin and gum arabic, the activity

of the catalases in the drops does not decline. For example, the data of Table 48 show that the enzymes in buffer solutions are tens of times more active than in the equilibrium liquid and hundreds of times in the drops.

Consequently, more favorable conditions are created for the enzymes in the drops than in ordinary buffer solutions used for evaluating the acitivity of catalase preparations. This may be due partly to the fact that in the drops the enzyme molecules are concentrated, whereas in the solution they are distant from each other.

It is possible that a definite spatial ordered arrangement of the molecules takes place in the drops. However, it is not known how they are packed or whether they are on the surface of the drops or inside them.

Coacervates containing enzymes and also the reactions performed by them are listed in Table 49.

When such systems are prepared not only the chemical composition of the substances but also the order in which they are combined are of great importance. For example, in order to form a coacervate in which RNA was split by ribonuclease, it is necessary to add RNA to gum arabic first, and then serum albumin. No coacervate drops are formed if this order is changed [262].

The influence of enzymes as protein compounds from the coacervates formed will /180
be studied in more detail on models represented in the works of Makovskiy and coauthors.

The proteolytic enzymes papain, pepsin and gastric juice, juice from the duodenum, and lipase, when added to the gelatin - gum arabic coacervate, disintegrated the coacervate drops [79, 222, 223, 436, 787, 789, 899, 939], and the enzymes in this case acted as chemical compounds which disintegrated the coacervates. If bile salts are added to such systems, the coacervates remain whole. According to Makovskiy, the protective role of bile salts apparently plays a definite part in protecting coacervate drops in organisms.

Enzymes can destroy coacervates by splitting their component parts. For example, such is the effect of polygalacturonidase on pectins of a coacervate obtained from proteins + pectin [939].

In addition, in studying coacervates both with enzymes included therein and with enzymes added to systems already formed, a decrease in the rate of the enzyme reaction is sometimes observed as compared to their action in buffer solutions. This was demonstrated, for example, for β-amylase in a gelatin - gum arabic coacervate, and for α-amylase and urease in a protamine sulfate - gum arabic system. According to the arguments of Makovskiy, Vasu and others, the enzyme is adsorbed on the coacervate drops, and this causes a decrease of its concentration in the equilibrium liquid and a slowing down of the reaction.

In some cases, the decrease of the reaction rate is due to a specific influence of the substances entering into the composition of the coacervate. For example, protamine decreases the activity α-amylase, but at the same time accelerates the action of urease [222].

The modeling of individual enzomatic processes has shown the fundamental possibility of carrying them out in coacervates and other enzyme reactions.

Table 49

COACERVATES CONTAINING ENZYMES AND MODELED ENZYME REACTIONS

No.	Composition of coacervate and enzyme reaction	pH	Temperature, °C	Reference
1	Sickle protamine sulfate - starch - gelatin - bacterial α-amylase of thermophilic variant of Clostridium pasteurianum (starch + H_2O → dextrine)	6.8-7.0	50	[261]
2	Gelatin - gum arabic - soybean β-amylase (starch + $+H_2O$→ maltose)	4.82	42	[122]
3	RNA - gum - serum albumin - pancreas ribonuclease (RNA + H_2O → nucleotides)	4.3	37	[262]
4	RNA - histone - pancrease ribonuclease (RNA + + H_2O → nucleotides)	9.5	37	[325]
5	RNA - pancrease ribonuclease (RNA $+H_2O$)→nucleotides)	4.5-5.5	37	[289]
6	Potassium oleate - RNA - pancrease ribonuclease (RNA $+H_2O$)→ nucleotides)	9.5	37	[331]
7	Serum sodium albumin arabinate - papain (protein + + H_2O → peptides + amino acids)	3.5	38	[211]
8	Glucose-1-phosphate (starch - seed) - NaF, gum - potato phosphorylase - histone (glucose-1-phosphate→ → starch + phosphoric acid	6.0-6.2	18-22	[149, 151, 260]
9	RNA - histone - ADP - polynucleotide phosphorylase of Micrococcus lysodeikticus (adenosine diphosphate → polyadenylic acid + inorganic phosphorus)	9-10	37	[265, 266, 326]
10	Mixture: nADP + polynucleotide phosphorylase M. lysodeikticus + histone → (polyadenylic acid → histone → coacervate)	9.5	37	[271]
11	Serum albumin - histone - bacterial dehydrogenase - NAD - H_2 - 2-6 DCPI (NAD - H_2 + 2 - 6DPI → NAD+ + 2 - 6DCPI - H_2)	7.0-7.4	18-22	[270]
12	Histone - gum arabic - fungus polyphenol - oxidase - paracresol - tyrosine - pyrocatechol - ascorbic acid - 2-6 DCPI. Oxication and reduction ——→ quinones, dehydroascorbic acid, 2-6 DCPI - H_2	6.8	16-25	*
13	Histone - lecithin - fungus polyphenol oxidase - tyrosine - pyrocatechol - ascorbic acid - 2-6 DCPI. Oxidation and reduction ——→ quinones, dehydroascorbic acid, 2-6 DCPI - H_2	5.5	16-25	*
14	Gelatin - gum arabic - liver catalase + H_2O_2 ($2H_2O_2$ →$2H_2O$ + O_2)	4.27	43	[143]
15	Sickle protamine sulfate - gelatin - liver catalase ($2H_2O_2$ → $2H_2O$ +O_2)	6.0	41-43	[143]
16	Sickle protamine sulfate - gum arabic - bacterial catalase of M. lysodeikticus ($2H_2O_2$ → $2H_2O$ + O_2)	6.0	25-43	[143]
17	Sickle protamine sulfate - gelatin - bacterial catalase of M. lysodeikticus ($2H_2O_2$ → $2H_2O$ + O_2)	8.8-9.0	41-43	[143]

*Unpublished data obtained by author together with Shurygina.

The study of enzymes in coacervate systems has begun recently. Data on this problem are scarce. However, even now the prediction can be made that the coacervate state of proteins and carbohydrates in the organism plays a definite role in the regulation of enzyme reactions.

In the case under consideration, of particularly great importance is the concentration of enzymes in the drops or their migration from the drops into the surrounding liquid. The specific character of the distribution and action of enzymes in coacervates depends on both the chemical composition of the coacervate and the nature of the enzyme.

The realization of catalytic enzyme reactions in coacervate drops transforms them into open systems. This is important for their development and also for the differentiation of biological processes in protoplasm. /181

CONCLUSION

There is a definite resemblance between the behavior of coacervates and protoplasm. In order to make a more detailed study of this problem, Bungenberg de Jong obtained coacervates included in hexagonal prismatic cells whose walls consisted of celloidin which simulated the envelope of plant cells, and demonstrated the morphological changes, which closely resembled the cases arising in cells.

Such models were used to study the action of a constant electric field, temperature, pH, nonelectrolyte dyes and other compounds on coacervate systems. The formation of a pulsating vacuole was demonstrated with toluidine blue [534, 535], and the penetrability of salt ions and their influence on the structure of coacervates were also studied [514, 515, 523, 525, 562, 567, 568, 571]; in many cases, this aided in the explanation of incomprehensible changes in the permeability of the envelopes of plant cells immersed in solutions of mineral salts of different concentrations. Usually, small amounts of salt cause a densification of coacervates. A further increase leads to swelling as a result of a redistribution of charges, and this is followed by an increase in the permeability of coacervates. A similar phenomenon was observed in the study of the permeability of the tonoplast of Sphaeroplea moths by immersing the cells in solutions of salt of various concentrations [701].

By studying ribosomes and coacervates of histone - RNA, Tashiro and Liebl showed that different concentrations of $MgCl_2$ act in the same direction on both ribosomes and coacervate drops [773, 923-926].

The structure of lipoprotein protoplasmic membranes is essentially the same as that of coacervate drops [481, 780, 824, 916, 917].

In addition to a certain resemblance in behavior between artificial models and proto plasm, the formation of natural coacervate drops in cells was demonstrated [240-242, 458, 668, 827].

When transferred into water, protoplasma breaks down into coacervate droplets The property of protoplasm ready to give coacervates may be regarded as a method /182 of unmixing, separation and concentration of necessary compounds from their dissolution and dispersion in the surrounding medium, has the creation of additional interfaces for the realization of enzyme reactions [187, 220, 285, 505, 687, 767, 768].

In protoplasm, coacervates are formed as a result of chemical enzyme reactions and participate in the complex mechanism of these processes.

One can find many different instances of separation of substances in protoplasm and vacuoles in the form of coacervate drops. This is particularly frequent in meristematic tissue during the development of cells. Young cells contain many proteins and nucleic acids which readily give coacervate drops [322, 357, 460, 555, 696, 697, 758, 868, 894].

Phosphate-oleic coacervates are of major importance in the absorption of fats. The transport of nutrients from the blood to glia cells takes place in the form of globules of a coacervate nature [727].

According to De Kuthy [761], coacervates containing cholesterol take part in the formation of gall stones. The formation of coacervates with anthocyans and other compounds was shown by Kuster [760].

Coacervate drops are also formed in pathological cases under unfavorable conditions.

For example, Dufrenoy [665-666] observed that polyphenol coacervates are formed during the development of a plant on a culture medium without Zn in the vacuoles of orange root cells. The presence of coacervate structures in protoplasm does not contradict the present day concept of the reticuloendothelial network of protoplasm as a system of channels and bubbles surrounded by lipoprotein membranes.

In no case do we place an equality sign between artificial coacervates and cell protoplasm. Unfortunately, there are no primitive free-living forms of one-celled animals available. For this reason, it is necessary to compare coacervates with so-called protozoa and cells of higher organisms. Modern Protista are already highly differentiated forms of life. Even the free-living microplasms, which are one-tenth the size of bacteria, are fairly complex [237].

Since coacervates can be obtained from the same chemical compounds which enter into the composition of live organisms, some properties of the protoplasm of living organisms and coacervates can also be the same [129, 173].

These properties of coacervate models find increasingly wider applications not only in the elucidation of processes occurring in the protoplasm but also for practical purposes. /183

Practical Application of Coacervates. The practical use of coacervates is based on the following properties of coacervate systems:

1) concentration of compounds from dilute solutions in small volumes;

2) ready separation of the layer for drops, where the molecules of dissolved compounds are concentrated;

3) possibility of obtaining drops of the desired size, shape and diverse chemical composition.

Coacervate drops having a definite refractice index are used for checking physical instuments, for example in the case of interference microscopes [648]. In this case, drops containing gelatin are most frequently employed.

Protein and coacervate drops 10-20 mμ in diameter consisting of hemoglobin,

gamma-globulin, serum albumin, histone and gelatin are recommended by Kelly and Carlson as standard models for quantitative reactions for amino acids (arginine, tyrosine, etc.) in cells, and also for measuring the ability of protoplasm to absorb dyes [736]. Coacervates can be used for fractionating various compounds [762, 910].

The separation of a mixture of blood serum proteins by means of coacervation was carried out by Motet-Grigoras. A gum arabic solution was added to serum at pH 3-4. It was found that only albumins participate in the formation of coacervate drops with gum arabic. The drops are readily separated from the mixture by centrifuging, and globulins remain in the serum [822, 823].

When developed further, the method can be used for elucidating the relationships between albumins and globulins not only in normal blood but also in pathological processes.

Matetskiy prepared coacervate drops from alkyl amide derivatives and various dyes for staining tissues. They gave very good results, much better than the usual methods of staining [224].

Coacervates have found particularly broad applications in the pharmaceutical industry in the preparation of drugs. Russell [878] coacervated aspirin, riboflavine, castor oil, etc., together with gelatin and Na_2SO_4. Coacervation is used for fractionating gelatin [910]. Gelatin with a molecular weight of 20,000 is used particularly frequently as the high-molecular component. Gelatin forms coacervates with different compounds. Tannin, alkaloids (morphine, etc.) and enzymes (pancreatin, antibiotics, certain oils, etc.) which form drops with gelatin are concentrated in this manner [627]. /184

Thus, the basic property of coacervation most thoroughly discussed in the present monograph is the concentration of compounds in coacervate drops.

All organisms, including Protozoa, differ markedly from the medium surrounding them not only in chemical composition but also in the concentration of the compounds comprising them. The coacervate systems examined in the book consisted chiefly of compounds formed in organisms, i.e., biogenically.

Many of the natural compounds were prepared from simple molecules under so-called abiogenic primitive conditions existing on the earth prior to the appearance of life.

If the ocean is regarded as the cradle of life, then one of the mandatory conditions for the appearance of life was the combination of molecules and their separation from the surrounding water in a given closed space. It is possible that during the initial stages, this took place in the form of coacervate drops. Their formation requires 0.01-0.001% of substances. It is postulated that the amount of organic compounds in /185 the primeval ocean considerably surpasses the coacervation limit.

As examples, Tables 50 and 51 present data on the concentration and content of the dry mass of substances and the volume occupied by them in various representatives of organisms and in coacervate drops. In compiling these tables, we made use of appropriate literature sources and also carried out the necessary calculations.

It follows from the data of Tables 50-51 that the main role among all the compounds is played by macromolecules, which determine the form of the organism and take an active part in its life.

Table 50

CONTENT AND CONCENTRATION OF THE DRY MASS OF SUBSTANCES IN VARIOUS SPECIMENS

Specimen	Diameter, cm	Concentration of dry mass %	Weight of dry mass, %
Mycoplasm	$2{,}5\cdot10^{-5}$	—	—
Bacteria	$2{,}5\cdot10^{-4}$	25—30	$2.5\cdot10^{-12}$
Mammalian cells	$2{,}5\cdot10^{-3}$	10—25	$2.1\cdot10^{-9}$
Ameba	$1\cdot10^{-2}$	10—15	$7{,}8\cdot10^{-8}$ ($1\cdot10^{-7}$)
Coacervate drops	$2{,}42\cdot10^{-4}$— —$1.01\cdot10^{-2}$	7—34	$2.5\cdot10^{-12}$— —$3.5\cdot10^{-8}$

Table 51

SPACE OCCUPIED BY MACROMOLECULES (PROTEINS + ENZYMES + NUCLEIC ACID) IN VARIOUS SPECIMENS

Specimen	Volume of specimen, cm^3	No. of macromolecules	Volume occupied by macromolecules, cm^3
Mammalian cells	$8{,}5\cdot10^{-15}$	18750	$2{,}09\cdot10^{-15}$
Bacteria	$8{,}5\cdot10^{-12}$	$4\cdot10^{7}$	$4{,}4\cdot10^{-12}$
Mammal cells	$8{,}5\cdot10^{-9}$	$3{,}6\cdot10^{10}$	$4\cdot10^{-9}$
Ameba	$5{,}2\cdot10^{-7}$	$1{,}2\cdot10^{12}$	$1{,}3\cdot10^{-7}$
Coacervate drops	$7{,}4\cdot10^{-12}$— —$5{,}3\cdot10^{-7}$	$5\cdot10^{7}$— —$7{,}2\cdot10^{11}$	$5{,}5\cdot10^{-12}$— —$7{,}9\cdot10^{-8}$

Macromolecules occupy a considerable part of the total volume of cells. Enzymes constitute approximately 10% of all protein Molecules. If one calculates the number of enzymes and also other macromolecules participating in the various chemical conversions, one finds that the process of protein synthesis is first in the consumption of macromolecules. This process requires a considerable portion of space. The synthesis of a single macromolecule of protein (molecular weight 30,000) made up of 20 different amino acids, involves over 50 macromolecules belonging to enzymes, different RNA and ribosomes. The end product of the synthesis is also a macromolecule. Much less space is required by the hydrolysis of proteins, polysaccharides and nucleic acids. In this case, the macromolecule splits another macromolecule into low molecular compounds.

All these comparisons are preliminary. For example, no account is taken here of the activity of the enzyme molecule, expressed by so-called number of turns adopted in the old classification of enzymes.

The number of turns shows the ability of the enzyme molecule to return to its initial state in a given period of time after its participation in the reaction [176].

One of the most active biocatalysts is catalase. The reaction of H_2O_2 decomposition

by catalase proceeds almost instantaneously, after which the molecule can again act further.

Obviously, such a high activity and the presence of three acting catalytic centers in a central molecule [671] considerably decrease the space required for the reaction.

Nor have the period of existence of enzyme molecules and a number of other factors been considered in our calculations. Obviously, in addition to the portion of the volume occupied by the macromolecules of the reacting substances, free space is also necessary for separating the reactions from each other and also for transferring the substances to the sites where they are consumed, i.e., a space filled with water and low molecular compounds is required.

The presence of not only high and low molecular compounds but also water in coacervate drops makes it possible to reproduce enzyme reactions in the drops. /186
Knowing the volumes and quantity of molecules necessary for the reaction, one can approach the creation of model coacervate systems in which multistage enzyme reactions leading to the formation of new compounds can take place.

The liquid nature of the drops, the diversity of their shape and chemical composition, the separation from the surrounding medium, and at the same time the interaction with this medium and also the many physical and chemical properties making it similar to protoplasm account for the fact that coacervate drops constitute very suitable models whose use along with other objects can elucidate and reproduce many phenomena characteristic of protoplasm and provide an approach to their regulation for solving the most important problem in biology, i.e., the artificial synthesis of live matter.

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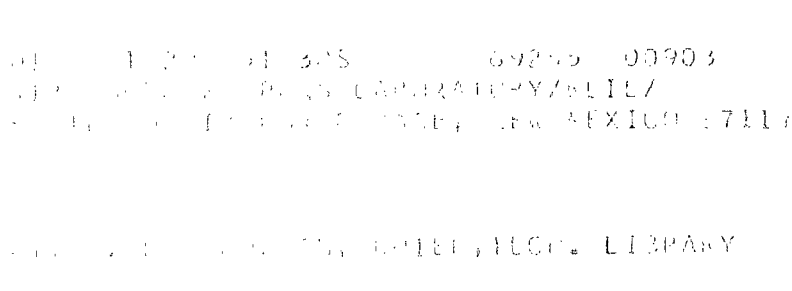

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